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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	
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U.S APPLICATION NO. (IF KNOWN, SEE 37 CFR)	
10/009085	

INTERNATIONAL APPLICATION NO	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US00/15565	5 June 2000	4 June 1999

TITLE OF INVENTION

SYSTEM AND METHOD FOR PRENATAL DIAGNOSTIC SCREENING

APPLICANT(S) FOR DO/EO/US

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Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information.

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include itens (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☒ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

Postcard Acknowledgement

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
10/009085	PCT/US00/15565	6033-001

24. The following fees are submitted:.		CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :			
☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO	$1040.00		
☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO	$890.00		
☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO	$740.00		
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4)	$710.00		
☒ International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4)	$100.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =		$100.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).		$0.00	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	10 - 20 =	0	x $18.00	$0.00	
Independent claims	1 - 3 =	0	x $84.00	$0.00	
Multiple Dependent Claims **(check if applicable)**			☐	$0.00	
TOTAL OF ABOVE CALCULATIONS =				$100.00	
☒ Applicant claims small entity status. See 37 CFR 1.27). The fees indicated above are reduced by 1/2.				$50.00	
SUBTOTAL =				$50.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +				$0.00	
TOTAL NATIONAL FEE =				$50.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).** ☐				$0.00	
TOTAL FEES ENCLOSED =				$50.00	
				Amount to be: refunded	$
				charged	$

a. ☒ A check in the amount of **$50.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **18-2000** A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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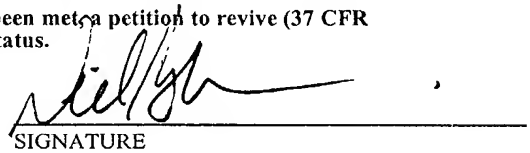

SIGNATURE

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31,872
REGISTRATION NUMBER

December 4, 2001
DATE

WO 00/75647 PCT/US00/15565

SYSTEM AND METHOD FOR PRENATAL DIAGNOSTIC SCREENING

Cross-Reference to Related Inventions

This patent application is a continuation-in-part of United States Provisional Patent Application Serial No. 60/137,692 filed June 4, 1999, from which priority is hereby claimed.

Background of the Invention

The present invention relates to a method for characterizing cells using a combination of histochemical staining, antibody labeling and fluorescence *in situ* hybridization (FISH). More particularly, the present invention relates to a method for characterizing cells using sequential histochemical staining, antibody labeling and FISH to provide data about morphology, antigenic sites as well as ploidy of cells. The use of fluorescent tags in molecular biology and genetics is expanding rapidly. This increased use of fluorescence necessitates the development of a new generation of imaging systems that are as effective with low light images as with the traditional light microscopy. Among the requirements for fluorescence imaging systems are:

- The ability to quantitate and compare signal intensity.
- The ability to scan and search for the presence of a rare signal, then relocate and return to that specific signal.
- The ability to count and track the number of cells exhibiting specific fluorescent signal characteristics.

The present invention also includes an imaging methodology and control software which is useful in routine fluorescent imaging of FISH labeled cells. Fluorescent labels are used as reporter molecules for a number of different applications. Fluorescent *In Situ* Hybridization (FISH) techniques are used to detect and identify specific chromosomes and chromosome regions. Gene expression products are identified in whole cells through the use of green fluorescent protein (GFP). Using two model systems, the IMAGESCAN Image Analysis Software System has been used to locate and capture specific fluorescent images while scanning a slide, return to the specific slide location and allow visual

confirmation, either immediately or at a later time. One model system is for detection of small fluorescent spots, such as those found with FISH of specific chromosomes. Male blood was spiked into female peripheral blood at a level of 0.1% v/v. Lymphocytes were separated, centrifuged onto slides and labeled by FISH using X and Y chromosome probes. A set of parameters defining the intensity and size of a fluorescent signal of interest was selected. The slide was automatically scanned. Fields containing images matching the parameter settings were captured. At completion of the scan, captured fields were reviewed and verified for the presence of fluorescent cells. Slides were prepared from peripheral blood and cell nuclei were labeled with DAPI. Parameters were set for the detection of whole cells. Slides were scanned and verified as described above. Final reports were prepared including tables summarizing the number of chromosomes or nuclei captured and an image gallery of captured fields.

Invasive prenatal genetic diagnosis is an integral part of obstetrical practice. Currently, the most common reason for offering prenatal genetic testing is to evaluate women at increased risk for fetal chromosome abnormalities. Chromosomal aneuploidies involving chromosomes 13, 18, 21, X and Y account for up to 95% of all liveborn chromosomal aberrations resulting in birth defects (Whiteman DHA, Klinger K., "Efficiency of rapid in situ hybridization methods for prenatal diagnosis of chromosome abnormalities causing birth defects," *Am J Hum Genet* 49, A127-129 (1991), and up to 67% of all chromosomal abnormalities, including balanced translocations (Klinger K, et al., "Rapid detection of chromosome aneuploidies in uncultured amniocytes by using fluorescence in situ hybridization (FISH)," *Am J Hum Genet* 51, 55-65 (1992) Prenatal chromosome analysis is routinely offered to women considered to be at high risk of having a child with a chromosomal abnormality. The most common risks are advanced maternal age; high-risk levels of maternal serum alpha-fetoprotein (MSAFP) or indicative Triple-Test results; the combination of MSAFP, beta-HCG (human chorionic gonadotrophin), and estriol, and abnormal ultrasonographic findings and family history.

The incidence of Down syndrome is 1 in 800 liveborns; and prenatal testing is recommended for women 35 years of age or more since the frequency of occurrence is 0.5% at 35 years of age and increases further thereafter (Evans et al., 1992). However, as there are far more pregnancies that occur to younger women, between 50 and 70% of Down syndrome babies are born to women younger than 35 years of age even though younger mothers are individually at lower risk. (Thompson, MW, McInnes, RR, Willard

HF. 1991. Thompson & Thompson Genetics in Medicine, Fifth Edition. WB Saunders, Philadelphia. p. 224; Simpson JL, Elias S. 1993. Isolating fetal cells from maternal blood. Advances in prenatal diagnosis through molecular technology. *JAMA* 270, 2357-2361 (1993); Haddow JE, et al.. "Prenatal screening for Down syndrome" Simpson JL, Elias S, editors, Essentials of Prenatal Diagnosis. Churchill Livingstone, New York, 185-220 (1993).

The currently accepted procedures for the diagnosis of fetal genetic abnormalities include chorionic villus sampling (CVS) and amniocentesis. Traditionally, amniocentesis and CVS have been routinely offered to women who are 35 years of age or older at delivery (Hook EB. "Rates of chromosome abnormalities at different maternal ages," *Obstet Gynecol* 58, 282-285 (1981)), because the risk of a trisomy (1 in 180) for this age group is about equal to the risk of miscarriage from the procedure (about 1 in 200). Among pregnant women undergoing amniocentesis and CVS, the rate of miscarriage is increased by 0.3 to 0.5% and 1 to 4% above background levels, respectively (Buselmaier W, Tariverdian G. (1991). Humangenetick. Berlin-Heidelberg-New York: Springer-Verlag; Murken J, Cleve H. (1996). Humangentick. 6. Aufl. Murken J, Meitinger T. Bearbeiter. Stuttgart: Ferdinand Enke.). The rate is higher for those patients who require multiple catheter or needle passes. Complication rates later in pregnancy are also comparable between the two methods and are significantly increased above background levels. CVS has been associated with increased risk for congenital anomalies, specifically transverse limb reduction defects, and oromandibular-limb hypogenesis (Hsieh FJ, et al. "Limb defects after chorionic villus sampling," *Obstet Gynecol* Jan 85(1), 84-88. (1987).

Efforts to develop clinical protocols to identify women at increased risk for fetal abnormalities had led to the use of maternal serum levels of alpha-fetoprotein, human chorionic gonadotrophin, unconjugated estriol and pregnancy-associated placental protein A, which are used to obtain an adjusted risk for fetal Down syndrome (Bogart MH, Pandian MR, Jones OW. 1987. Abnormal maternal serum chorionic gonadotrophin levels in pregnancies with fetal chromosome abnormalities. *Prenat Diagn* 7,623-630; Cuckle HS, et al., "Combining inhibin A with existing second-trimester markers in maternal serum screening for Down's syndrome,". *Prenat Diagn* 16, 1095-1100 (1996). However, serum biochemical marker screening is not without uncertainty and identifies only 60-70% of fetuses with Down syndrome with a 5% false positive rate (Shulman LP. 1992. Perspectives on counseling in maternal serum alpha-fetoprotein screening. In Maternal

Serum Screening for Fetal Genetic Disorders (Elias S, Simpson JL, eds.) Churchill Livingstone, New York, pp. 121-130). Ultrasound has also been used, either alone or in combination with selected maternal serum analytes, to identify women at increased risk for fetal abnormalities. However, both maternal serum analyte analysis and ultrasound suffer form poor sensitivity or specificity. Accordingly, a method using fetal cells form maternal blood could potentially better identify women at increased risk for fetal chromosome abnormalities and genetic disorders and would be characterized by an improved sensitivity and specificity compared to currently available screening protocols. This would serve as a more accurate protocol for fetal risk assessment and, potentially in the future, prenatal diagnosis.

During pregnancy, multiple fetal cell types cross the placenta and enter maternal circulation. The cell types that can be isolated from maternal blood for possible use in prenatal diagnostic testing include trophoblasts, lymphocytes, and erythroblasts or nucleated red blood cells. Trophoblasts were the first cells to be identified, but are not suitable for prenatal diagnosis because of their heterogeneous genetic nature and rapid clearance by the maternal pulmonary circulation (Steele CD, et al., "Prenatal diagnosis using fetal cells isolated from maternal peripheral blood: a review" *Clin Obstet and Gynecol* 39, 801-8136 (1996); Bianchi DS. "Current knowledge about fetal blood cells in the maternal circulation" *J Perinat Med* 26, 175-185 (1998). Fetal lymphocytes are known to persist in the maternal circulation for 5 years or longer (Ciaranti A., et al., "[Survie de lymphocytes foetaux dans le sang maternal post-parum]" *Schwetz Med Wochenschr* 107, 134 (1977); Schroder J, Tiilikainen A, de la Chapelle A. 1974. "Fetal leukocytes in the maternal circulation after delivery: I. Cytological aspects," *Transplantation* 17, 346 (1974). Post-partum persistence of fetal lymphocytes leads to the potential for diagnostic error arising from genetic analysis of cells originating from a previous pregnancy. Fetal NRBC first appear in the maternal circulation at six weeks of gestation as the liver develops (Hamada H, et al., "Fetal nucleated cells in maternal peripheral blood: frequency and relationship to gestational age," *Hum Genet* 91, 427-432 (1993); Liou JD, et al., "Fetal cells in the maternal circulation during the first trimester in pregnancies," *Hum Genet* 19, 427 (1993). NRBC appear to be best suited for prenatal diagnosis, due to their short half-life in the maternal circulation. Barring recent spontaneous abortion, NRBC should not be present from a prior pregnancy (Elias S, et al. "Isolation of fetal nucleated red blood cells from maternal blood: persistence of cells from

prior pregnancy is unlikely to lead to false positive results." *J Soc Gynecol Invest 3 (Suppl)* 359(A) (1996).

Significant amounts of fetal DNA have been detected in maternal blood using PCR techniques for the measurement of sequences specific to the Y-chromosome. Numbers of fetal cells have been estimated from PCR measurements of fetal DNA in either whole blood (i.e., no enrichment) or samples enriched for fetal cells. Enriching for fetal cells increased the estimated range from 19 - 6,000 to 225 - 22,500 fetal cells per 15 ml of maternal blood (Bianchi DW, Flint AF, Pizzimenti, MF, Knoll JHM, Latt SA. 1990. Isolation of fetal DNA from nucleated erythrocytes in maternal blood. PNAS 87, 3279-3283; Adinolfi M, Camporese C, Carr T. 1989. Gene amplification to detect fetal nucleated cells in pregnant women. *Lancet* 2, 328-329; Ganshirt D, et al. "Enrichment of fetal nucleated red blood cells from the maternal circulation for prenatal diagnosis: Experiences with triple density gradient and MACS based on more than 600 cases," *Fetal Diagn Ther* 13, 276-286 (1997); Nakagome Y, et al. "Absence of fetal cells in maternal circulation at a level of 1 in 25,000" Am J Med Genet 40, 506-508 (1991); Kao, SM, et al. "Analysis of peripheral blood of pregnant women for the presence of fetal Y chromosome-specific ZFY gene deoxyribonucleic acid sequences" *Am J Obstet Gynecol* 166, 1013-1019 (1992); Bianchi DW, et al. "PCR quantitation of fetal cells in maternal blood in normal and aneuploid pregnancies," *Am J Hum Genet* 61, 822-829 (1997); Lo YMD, et al. 1998. "Quantitative analysis of fetal DNA in maternal plasma and serum: implications for non invasive prenatal diagnosis." *Am J Hum Genet* 62, 768-775 (1998).

Since NRBC are short-lived cells of limited replicative capacity, they are ideal candidates for prenatal diagnostic testing. Sekizawa and colleagues (1996) obtained a prenatal diagnosis of Duchenne muscular dystrophy, a single gene disorder, and also were able to accurately determine fetal RhD status. A drawback of the approach is that the gene of interest must be absent from the maternal genome, or the fetal NRBC must be distinguished from the maternal cells. Although obtaining a full karyotype from fetal erythroblasts has not been reported, expansion of fetal erythroid progenitors has been achieved with cells isolated from the CFS and the sex determined by PCR (Wachtel SS, et al. 1996. "Fetal cells in maternal blood: recovery by charge flow separation," *Hum Genet* 98,162-166 (1996). At the present time, numerous authors (Oosterwijk JC, et al., "Strategies for rare-event detection: an approach for automated fetal cell detection in maternal blood," *Am J Hum Genet* 63, 1783-1792 (1998); Jansen et al., "The use of in

vitro expanded erythroid cells in a model system for the isolation of fetal cells from maternal blood," *Prenat Diagn* 19, 323-329 (1999), Wang JY, , et al. "Fetal nucleated erythrocyte recovery: fluorescence activated cell sorting-based positive selection using anti-gamma globin versus magnetic activated cell sorting using anti-CD45 depletion and anti-gamma globin positive selection" *Cytometry* 39, 224-230 (2000); Wachtel et al., (1996); Bianchi, (1997) have examined the fetal cells isolated from maternal blood with fluorescence in-situ hybridization (FISH) to detect the most common chromosomal aneuploidies (X, Y, 13, 18, and 21).

There has been some disagreement in the literature regarding the concentrations of NRBC in the maternal circulation. Using FISH and PCR to estimate number of fetal cells in maternal blood, Hamada (1993) found that both methods showed less than 1 fetal cell per 100,000 nucleated maternal cells in the first trimester and about 1 fetal cell per 10,000, maternal cells at term. Other researchers have used PCR amplification of DNA sequences and found low frequencies of fetal cells, with estimates from less than 1 per 5000 to 1 per 10^8 maternal cells (Bianchi et al., 1990; Adinolfi et al., 1989). Liou et al. (1994) studied 19 women with male fetuses, drawing blood at weekly intervals starting early in the first trimester. At six weeks, fetal cells were identified in one woman; at 9 weeks, in 15/19 of the women; and at 12 weeks, in all of the women. Thomas et al. "The time of appearance and disappearance of fetal DNA from the maternal circulation." *Prenat Diagn* 15, 641 (1995) demonstrated that Y-chromosome DNA could be detected in fetal cells as early as 4 weeks, 5 days post-conception (using in vitro fertilization to provide a precise date of conception). In all patients studied, fetal genetic material was detected in the maternal circulation by 7 weeks of gestation.

At 11 weeks, approximately 10% of fetal erythrocytes in the fetal circulation are nucleated. Given 50,000 NRBC/μl of fetal blood at 11 weeks of gestation (Bianchi et al., 1990), a 10 μl transfer across the placenta into a maternal blood volume of 5 liters translates into 1,500 NRBC/15 ml maternal blood. Because one ml of maternal blood also contains 5×10^9 maternal erythrocytes and 7×10^6 maternal leukocytes (Diem K, Lentner C, eds. Documenta Geigy Scientific Tables, Seventh Edition. Ciba-Geigy Limited, Ardsley, NY, 1970), the expected ratio of NRBC to maternal blood cells would be 1 per 5×10^7. Maternal peripheral blood contains both fetal and maternal nucleated erythrocytes (Slunga-Tallberg A, et al. "Maternal origin of nucleated erythrocytes in peripheral venous blood of pregnant women," *Hum Genet* 96, 53-57 (1994) and Slunga-Tallberg A, et al.

"Nucleated erythrocytes in enriched and unenriched peripheral venous blood samples from pregnant and nonpregnant women," *Fetal Diagn Ther* 9, 291-295 (1995)). Studies by von Eggeling et al. (1997) and Wachtel et al. (1996) evaluating NRBC versus fetal cells report 30 to 35% of the NRBC are fetal in origin. In addition, conditions such as pre-eclampsia increase the relative proportion of fetal to maternal nucleated erythrocytes in maternal circulation (Holzgreve et al., "Disturbed feto-maternal cell traffic in pre-eclampsia,. *Obstet Gynecol* 91, 669-672.1998a). Also, aneuploid fetal cells also appear to occur in greater frequencies in maternal blood than do normal fetal cells (Bianchi, et al., 1997).

A number of biophysical methods have been used to enrich fetal cells from maternal blood: density gradient centrifugation, selective lysis, affinity adsorption to a solid matrix, fluorescent activated cell sorting (FACS), magnetic activated cell sorting (MACS) (both negative and positive), and charge flow separation (CFS). The use of any one method exclusively has not been completely successful in accomplishing the simultaneous (and frequently conflicting) requirements of recovery and purity. The FACS method requires expensive equipment and a specially trained operator (Martin et al., "Non-invasive fetal cell isolation from maternal blood,". *Br J Obstet Gynaecol* 105, 576-583 (1998). While FACS enriches fetal cells to a high level of purity, large numbers of contaminating cells limit the usefulness of the method by increasing sorting time and expense (Sekizawa et al., "Improvement of fetal cell recovery from maternal blood: suitable density gradient for FACS separation." *Fetal Diagn Ther* 14, 229-233 (1999). The magnetic selection methods, in combination with density gradient centrifugation, do not require expensive equipment and have demonstrated enrichment of fetal NRBC (Holzgreve et al., [Prenatal diagnosis with fetal cells in maternal blood: report of experiences in Basal]. [Article in German]. Schweiz Med Wochenschr 128, 1641-1645. (1998). Von Koskull et al., "Fetal erythroblasts from maternal blood identified with 2,3-bisphosphoglycerate (BPG) and in situ hybridization (ISH) using Y-specific probes," *Prenat Diagn* 15, 149-154. (1995) reported the range of recovery using negative magnetic selection with a monoclonal antibody to CD45 at 19 - 375 fetal cells from 15 ml of maternal blood. Higher recovery of 38 - 900 fetal cells from 15 ml of maternal blood was reported using positive selection by Campagnoli et al., "Noninvasive prenatal diagnosis: use of density gradient centrifugation, magnetically activated cell sorting and in situ hybridization," *J Reprod Med* 42, 193-199. (1998). Even though NRBC can be enriched by either FACS, or the use of magnetic selection, a sizable number of maternal cells still

co-enrich with the fetal cells. Fetal cell loss also varies to a great extent depending on the enrichment procedure (Holzgreve et al., 1998).

Using the CFS, the range of NRBC recovery has been reported at 28 – 22,500 NRBC per 15 ml of maternal blood in 225 samples (Wachtel et al., "Charge flow separation: quantification of nucleated red blood cells in maternal blood during pregnancy." *Prenat Diagn* 18,455-4631998). The rate of detection of fetal cells in maternal circulation was 67%. In another set of separations under locked conditions, NRBC were detected in 82% of samples (Section 3). Recently, using magnetic selection, detection rates of 80% were reported by Holzgreve et al., (1998). A multi-institutional study in the National Institute of Child Health and Human Development (NIFTY) using antibody based methods of FACS and MACS has determined the overall aneuploidy detection rate to be 50% (de la Cruz et al., "Low false-positive rate of aneuploidy detection using fetal cells isolated from maternal blood," *Fetal Diagn Ther* 13, 380 (1998). Although fetal cells are found in maternal blood using a variety of techniques, it is unclear whether all pregnant women have fetal cells in their blood.

The present Applicant, BioSeparations, Inc., through its Charge Flow Separation (CFS™), has shown that fetal cells can be recovered from the excess of maternal cells (Wachtel et al., 1996). The present invention utilizes an integration of three separate technologies for the enrichment, labeling and detection of rare cells into a single system termed the PRENATAL DIAGNOSTI SYSTEM (PDS™). These technologies consist of the CFS technology, specific detailed protocols for the sequential fluorescence in situ hybridization (FISH and ReFISH™) of enriched fetal cells, and IMAGESCAN™, a software package that allows the imaging, capture and analysis of labeled cells. The integration of the three separate technologies into a single system has been completed utilizing a model system containing fetal cells. In order for non-invasive diagnosis of aneuploidy to become the standard of care with the potential of replacing a significant number of amniocentesis or CVS procedures, a number of fundamental issues must be addressed. Research in the field of non-invasive prenatal diagnosis has been directed towards the identification and quantification of fetal cells in the maternal circulation at varying times during pregnancy and the optimization of methods for their isolation and purification.

A successful system for non-invasive prenatal diagnosis must, above all, provide accurate information and be safe for both mother and baby. An accurate diagnosis

requires that the maximum number of fetal cells be recovered by the system, and they must be at a high level of purity. In addition, the system should also provide rapid, timely information to both patient and clinician, be competitively priced with existing tests, and consist of a user-friendly process which allows operator input for critical steps and decisions. However, for this approach to be accepted as common practice, the fetal NRBC must be enriched using the technologies that have the best attributes of recovery and purity possible in order to obtain the maximum sensitivity and specificity for clinical diagnosis.

Summary of the Invention

According to conventional methodologies, the FISH protocol is performed first, followed by histochemistry/immuno-histochemistry. However, during the FISH protocol there is loss of morphological and biochemical features which makes histochemistry/immuno-histochemistry results difficult to interpret. The present invention provides a method for labeling cells using precipitable substrates and histochemical stains and scanning under bright field microscopy to locate labeled cells. Under bright field microscopy, the slides are scanned using IMAGESCAN software (BioSeparations, Inc., Tucson, Arizona) described in greater detail in U.S. Patent No. 4,592,089, which is hereby incorporated by reference, images of labeled cells are digitally captured and the location of each labeled cell is identified according to its coordinates on the slide and linked to the image. After digital scanning, chromosome are then labeled by FISH with CHROMAHYB 600 (BioSeparations, Inc., Tucson, Arizona) and DNA probes. FISH labeling procedures and the CHROMAHYB 600 reagent are more fully described in co-pending U.S. Patent Applications Nos. 08/949,243, filed October 10, 1997, 08/874,271 filed June 13, 1997, and 08/874,270 filed June 13, 1997 and PCT International Applications Publication Nos. WO 99/19511 published April 22, 1999 and WO 98/56955 published December 17, 1998, the disclosures of each are hereby expressly incorporated by refererence as teaching FISH protocols and hybridization buffers useful with the present invention.

Slides are returned to the microscope and using the stored information from the bright field microscopy, the IMAGESCAN software automatic repositions the slide to the stored image coordinates corresponding to the labeled cells to view the previously imaged cells for detection of the fluorescent FISH signals.

A model system using antibodies specific for subpopulations of leukocytes and FISH for the XY chromosomes demonstrates the utility of the method of the present invention. Potential applications include characterization of fetal cells in maternal cell populations, labeling of cancer cells followed by genetic characterization of the same cells, and monitoring of bone marrow transplants for XY/XX composition following opposite sex transplantation.

A system and method for prenatal diagnostic screening is also provided which integrates three major functions: enrichment, labeling and detection. Detection is facilitated by the IMAGESCAN software (BioSeparations, Inc. Tucson, Arizona) which interacts with hardware, including a microscope stage, camera, and optical filters, to scan a sample slide. The IMAGESCAN software creates files which are uploaded onto an internet web server which permits remote access, monitoring and control by any web-based browser.

Brief Description of the Figures

Figure 1 is a flow diagram illustrating the sequential steps of the inventive method for characterizing cells.

Figure 2 is a photomicrograph illustrating the use of DAPI as a histochemical label which distinguishes the NRBC from red blood cells by the blue fluorescence of the nucleus.

Figure 3 is a bright field photomicrograph illustrating monoclonal antibody labeling of NRBC and some leukocytes with monoclonal antibody 2F6.

Figure 4 is a photomicrograph illustrating cells labeled with fluorescent probes for the X and Y chromosomes.

Figure 5 is screen shot of the IMAGESCAN™ software which is used to return to the same slide location and capture corresponding images following different labeling techniques.

Figure 6 are graphs representing th effect of varying standard hybridization conditions in the standard FISH protocol.

Figure 7 is a screen shot of the FISHFINDER™ software after completion of a scan, illustrating the automatic graphical marking of fields containing cells of interest.

Figure 8 is two screenshots of output from the IMAGEFINDER software illustrating data obtained from detection of labeled slides.

Figure 9 are screenshots of the FISHFINDER software illlustrating slides prepared with female lymphocytes spiked with 0.1% male cells were scanned in search of SpectrumOrange labeled Y chromosomes. Images from the scan of mixed lymphocytes showing a Y containing cell in the midst of XX cells and the cell counting function of the system. Images are at 40 x using a triple pass FITC/TRITC/UV filter.

Figure 10 are screenshots from the FISHFINDER software illustrating cells expressing sgGFP™ (green fluorescent protein) imaged with a FITC filter at 20 x and Rev-sgBFP™ (blue fluorescent protein) imaged with a UV filter. SgGFP and Rev-sgBFP labeled cells were provided by Quantum BioTechnologies, Inc. Montreal, Canada.

Figure 11 is a schematic diagram of a Charged Flow Separator (CFS 100 and CFS 200) used in the present invention functionally illustrating operation of the Charged Flow Separator.

Figure 12 is a graph depicting the distribution of red blood cells (RBC), white blood cells (WBC) and nucleated red blood cells (NRBC) in fractions eluting from the Charged Flow Separator.

Figure 13 is a graph illustrating nucleated red blood cell (NRBC) recoveries in samples processed with the CFS 100 and the CFS 200.

Figure 14 is a photomicrograph field image and enlarged cell image depicting fluorescently labeled fetal NRBC, fetal RBC, maternal RBC and WBC obtained using the IMAGESCAN system.

Detailed Description of the Preferred Embodiment

During the process of characterizing cells it is frequently desirable to obtain information about both the genetic make up as well as the protein composition. In order to accomplish this, it is desirable to combine the techniques of immunohistochemistry with fluorescence *in situ* hybridization. Immunohistochemistry is used to label the proteins while *in situ* hybridization is used for identifying both DNA as well as RNA. One of the problems of the combined techniques is related to optimum fixation and the sequence of assays. The hybridization process uses chemistry and temperatures that frequently destroy the antigenic sites necessary for the immunohistochemistry. In addition, many of the

procedures used for immunohistochemistry render the cell nucleus resistant to penetration of the probe during hybridization.

Figure 1 is a flow diagram illustrating the method 10 of the present invention and its sequential steps in characterizing cells. The present invention comprises a method 10 which includes a sequence of labeling protocols that allow efficient monoclonal antibody (MoAb) labeling followed by FISH. DAPI (4',6-diamidine-2'-phenylindole dihydrochloride) is used for histochemical labeling of cell nuclei on a slide at step 12. Slides are scanned and images stored with linkage to their location on the slide at step 14. The histochemical label is followed by immunohistochemistry with an indirect monoclonal antibody reaction using an alkaline phosphatase substrate visible by bright field microscopy at step 16. Slides are returned to the microscope, the fields of interest relocated and bright field images captured at step 18. Slides are removed and FISH is performed at step 20. Slides are returned to the microscope for final imaging of the FISH results using fluorescence with the fields selected byased upon the immunohistochemical label being returned to by repositioning the slide using the inventive computer software IMAGEFINDER (BioSeparations, Inc., Tucson, Arizona), at step 22. The data is then analyzed using saved images in an image gallery at step 24.

Example 1

Slides

Slides were prepared using a Hettich Universal cytospin. 200,000 cells were centrifuged through 1 mL of buffer in a 12 mm circle. The sample on the slides was then fixed in 100% MeOH at room temperature.

Histochemistry

The samples on the slides were then treated with DAPI for labeling of DNA in nuclei. The samples were then scanned using fluorescence and a FITC/TRITC/UV filter.

Immunohistochemistry

Monoclonal antibody, 2F6, against the transferrin receptor on nucleated red blood cells (NRBC) was used to label cells. (Antibody provided by Washington University School of Medicine, St. Louis, MO.). Vector ABC-AP Kit with BCIP/NBT substrate was used for antibody labeling. Slides were re-examined using bright field microscopy.

FISH

Prior to FISH, slides were pre-treated according to the BioSeparations' CHROMAHYB™ instructions to remove cytoplasm. Slides were FISHed using the CHROMAHYB 600 Kit and Vysis X (SpectrumGreen) and Y (SpectrumOrange) satellite chromosome probes diluted 1:2 in CHROMAHYB buffer. Slides were denatured 2 min at 83° C and hybridized at 60° C for 1 hour, then washed at 60° C as per kit instructions. Slides were re-examined using fluorescence microscopy.

Imaging

Following DAPI labeling, slides were scanned by IMAGESCAN™ version 1.0.3 at 100x magnification. The user defines parameters that adjust threshold settings for accurate cell segmentation. Slide is automatically scanned. During the scan, continuous auto focus maintains optimal focal plane. Objects meeting the segmentation parameters are enumerated and marked. Image data is captured and linked to a color-coded graphic interface. This interface allows the user to retrieve, inspect, save or discard field images. The graphic enables relocation of specific fields after a slide has been removed for further processing. Cells from an image field are selected and saved to a gallery, linked through the graphic interface to the original study.

RESULTS

The data illustrated in Figures 2-4 provide different information about the nubleated red blood cells (NRBC). Figure 2, using DAPI as a histochemical label, distinguishes the NRBC from red blood cells (RBC) by the blue fluorescence of the nucleus. The autofluorescence of the surrounding hemoglobin distinguishes NRBC from white blood cells (WBC). In Figure 3 both NRBC and some leukocytes are labeled with the monoclonal antibody 2F6. Thus, NRBC can not be identified using the antibody data alone. Finally in Figure 4 the cells have been labeled with probes for the X and Y chromosomes. This provides data indicating which cells are male in origin and which are female. Utilizing data from histochemistry and immunohistochemistry, along with the FISH results, the gender of NRBC and white blood cells is assessed.

Figure 5 illustrates the IMAGESCAN™ software screens used to return to the same slide location and capture corresponding images following different labeling techniques.

The IMAGESCAN software enables repeated return to the same cells following sequential labeling methods. The first scan creates a study file with a graphic interface used to facilitate return to selected slide coordinates in subsequent steps. The first scan data is reviewed and fields of interest are marked. At that time, cells within a field can be selected and stored in a gallery linked to the study. After each sequential label, the slide is returned to the microscope, the coordinate positions saved in the graphic interface are used to relocate the marked fields. The corresponding cells of interest are selected and saved to the gallery.

The same cells can be sequentially labeled using histochemistry, immunohistochemistry and FISH methods. IMAGESCAN software enables the investigator to analyze identical cells, after labeling with different methods, through accurate return to the same fields of interest. Unique information useful for the characterization of cells can be obtained using sequential labeling methods for identification of morphology, protein composition and genomic make up.

IMAGESCAN Materials and Methods

Comparative Studies

Slides were prepared with Carnoy's fixed cells from male fetal liver. Fluorescence *in situ* hybridization assay conditions varied from the standard protocol as follows: Buffer 1 versus Buffer 2; Pre-treatment versus no Pre-treatment; Hybridization Time; Wash Stringency.

Rare Signal Location and Cell Counting

Peripheral blood lymphocytes (PBL) were separated from adult male and female anticoagulated blood by centrifugation using Histopaque 1077 and washed twice with phosphate buffered saline (PBS). Cells were counted and male cells were mixed into female cells at approximately 0.1%. Red cells were lysed and nucleated cells were fixed using ice cold Carnoy's (3:1 methanol:acetic acid). Slides were processed using the standard CHROMAHYB™ fluorescence *in situ* hybridization protocol. (BioSeparations, Inc., Tucson, AZ). Slides were scanned using IMAGEFINDER™ consisting of a fluorescent microscope, automated XYZ stage, three chip color CCD camera and FISHFINDER™ software.

Standard Hybridization Protocol

Pre-treatment - 5 min Pre-treatment solution (BioSeparations) followed by 1 min 70% Acetic Acid, followed by two successive cycles of 2 minutes each in 1:100 Wash Stock BioSeparations); 1 min each 70% EtOH, 85% EtOH, 100% EtOH.

Hybridization Cocktail/per slide - 7 μ L CHROMAHYB 600™ (BioSeparations); 1 μ L Calf Thymus DNA (Life Technologies); 0.5 μ L Alpha Satellite CEP X SpectrumGreen™ (Vysis); 0.15 μ L Satellite III CEP Y SpectrumOrange™ (Vysis); 1.35 μ L deionized H_2O.

Procedure - Add 10 μ L hybridization cocktail over cells and cover with 18 mm round cover glass; Denature at 83° C for 1.5 minutes; Hybridize at 60° C for 30 minutes. Wash at 60° C in Wash Solution (BioSeparations) using time, sequence and dilutions as follows: 1:80 3 min; 1:200 2 min; 1:80 30 sec; 4 x 1:400 30 sec each; Air dry; 10 μ L Mounting Medium (BioSeparations) and cover with 22x30 cover glass.

Observe and scan a portion of the slide using triple pass, FITC/TRITC/UV filter. Scanning parameters are set to count DAPI fluorescent cells and TRITC fluorescent spots.

Results

As illustrated in Figure 6, after analysis using the FISHFINDER™ software which was used to identify TRITC fluorescent signal in male fetal liver cells hybridized using variations of the standard protocol, the software determined intensity of the Y chromosome signal and saved the data in the Results Table. The data was exported to Excel and frequency distribution analysis was done using GraphPad Prism version 2.0. The chromosome intensity was compared under different assay variables to select optimal probe hybridization conditions. The four panels in Figure 6 demonstrate the application of the software for providing quantitative experimental data.

As illustrated in Figure 7, upon completion of a scan, the FISHFINDER™ software automatically provides a graphic marking those fields containing cells of interest. Each field can be reviewed, marked or discarded by working with the saved images or the actual image automatically relocated on the slide. The results are set forth in Table 1, below:

Table 1

Fields Counted	Cells Counted	Expected Y @ 0.1%	Counted Y	Actual % Y
50	2310	2.3	5	0.2%

Figure 8 are screen shots from the FISHFINDER software, demonstrating the output of information obtained for each spot detected using the FISHFINDER software.

Figure 9 are two screenshots from the FISHFINDER software illustrating the results of slides prepared with female lymphocytes spiked with 0.1% male cells were scanned in search of SpectrumOrange labeled Y chromosomes. The system automatically counted and recorded location, size and intensity data characterizing the cells and chromosomes. These data were used to calculate the number of Y chromosomes found as compared to expected. The images from the scan can be transferred into Word, Notepad, Paint or other software for printing. Images from the scan of mixed lymphocytes showing a Y containing cell in the midst of XX cells and the cell counting function of the system. Images are at 40 x using a triple pass FITC/TRITC/UV filter.

Finally in Figure 10, cells expressing sgGFP™ (green fluorescent protein) imaged with a FITC filter at 20 x and Rev-sgBFP™ (blue fluorescent protein) imaged with a UV filter. SgGFP and Rev-sgBFP labeled cells were provided by Quantum BioTechnologies, Inc. Montreal, Canada.

Conclusions

FISHFINDER™ software used on the IMAGEFINDER™ system provided quantitative results for comparison of assay variables and selection of optimal conditions for the most intense signal. A model system for locating rare cells demonstrated the ability of the FISHFINDER™ software to identify rare signal events. The system is able to select and count specific fluorescent signals based on selected parameters and report the data in tables and images.

The use of IMAGEFINDER™ with FISHFINDER™ software for image analysis with low light fluorescence microscopy has been demonstrated using model systems. These model systems represent commonly required needs for efficient use of low light fluorescent microscopy for detection of rare cells and labeling events and providing quantitative and qualitative comparisons of fluorescent images.

Thus, the present invention provides an imaging analysis tool for comparitive analysis of labeled cellular microscopic images and comparing immunohistochemical labels with fluorescence labels to yield an accurate and rapid scan of a sample for labeled cells. Additionally, the present invention provides a method for characterizing cells using a combination of histochemical staining, antibody labeling and fluorescence *in situ* hybridization (FISH).

Prenatal Diagnostic System (PDS)

The PDS protocol consists of three major tasks: enrichment, labeling and detection. Enrichment of the sample requires three discrete steps: 1) density gradient centrifugation; 2) Charge Flow Separation (CFS); and 3) magnetic positive selection of NRBC and lysis of mature maternal RBC. Labeling requires two additional steps: 4) fixation and slide preparation and 5), fluorescence in situ hybridization (FISH). The detection of fetal cells is the final task, and uses the IMAGESCAN program to 6) scan and 7) capture the labeled NRBC and its chromosomes.

A. Enrichment

Centrifugation is the first step in the enrichment process, and used to remove the bulk of the platelets and mature RBC without the loss of NRBC. Unfortunately, the losses during the centrifugation can be substantial, but this is balanced by a significant 150-fold increase in purity. Our current method yields about 56 ± 30% recovery of NRBC.

The second step in the enrichment process is charge flow separation. The CFS technology is an adaptation of free-flow electrophoresis, using a flow of buffer opposing the electrical charge to enhance the separation of cells. Briefly, the sample enters from the bottom of the separator, and is move in an upward direction by the flow of buffer. An electrical charge moves across the separator from the anode to the cathode, counterbalanced by a second flow of buffer from the cathode to the anode. Unwanted fluid gradients which would minimize separation are controlled by the use of stabilization media. In this system, cells are separated based on their charge-to-mass ratio. The CFS separator geometry is shown schematically in Figure 11, which depicts a CFS separator having a separation chamber sub-divided into a plurality of separation channels divided by membranes. A sample is input into a channel, an electrical field is applied, and a buffer flow having an orientation vector opposing the electrical field, known as a buffer

counterflow is applied to the sample. A plurality of fractions elute from the separation chamber containing the separated sample.

Among blood cells, RBC have the highest electrophoretic mobilities, and WBC the lowest. NRBC in maternal circulation originate in the fetal liver and represent various stages of development in the erythroid pathway. Thus, the NRBC are both RBC-like and WBC-like in their surface properties. Electrophoretically the NRBC should separate under the anodal shoulder of the RBC peak and under the cathodal shoulder of the WBC peak, depending upon its stage of erythroid maturation. Figure 12 illustrates a typical distribution profile of NRBC in relation to the RBC and WBC profiles separated with the CFS. Under these conditions, approximately 70% of the NRBC are located in CFS fractions 6 and 7 along with about 15% of the WBC and about 50% of the RBC.

Early Results with the CFS-100.

Since the NRBC is the fetal cell found in maternal blood preferred for non-invasive prenatal diagnosis, BioSeparations, Inc. has focused its research methods on this cell type. The early literature suggested a frequency of fetal cells in maternal circulation on the order of 1 per 10^6 to 10^8. A frequency of 1 fetal cell per 10^6 maternal cells would represent 75,000 fetal cells in 15 ml of maternal blood. At this level, most of the current technologies should be capable of isolating and identifying fetal cells. However, based on the work of others, as well as BioSeparations, Inc., the number of fetal cells per maternal cell is more on the order of 1 per 10^8 to 10^9, or less.

In early studies the CFS was used to purify NRBC that were subsequently subjected to FISH analysis for the Y-chromosome (Wachtel et al., 1996; Wachtel et al., 1998). In a blinded study, fourteen maternal samples were processed with the CFS, Carnoy's fixed and labeled with DNA probes to the X- and Y-chromosomes. Male gender was correctly identified in nine cases (90%) when 10,000 nuclei were scored. Because the true starting concentration of fetal cells in maternal cells was unknown, an inclusion criteria of at least 0.2% (1 NRBC/500 nucleated cells) was selected. In this study, the range of NRBC recovered from the CFS was 380 - 36,000 (mean 10,319). Assuming 30 - 35% of the NRBC are fetal (von Eggeling et al., 1997; Wachtel et al., 1996), then an average of greater than 3000 fetal cells were isolated in the samples analyzed.

Utilizing three different models of the CFS-100 separator and multiple research settings, 225 samples of maternal blood were processed and the NRBC per 15 ml sample

was estimated (Wachtel et al., 1998). The reported range of NRBC recovered was 38 - 22,500. NRBC were detected in maternal circulation in 67% of the samples.

A more recent study group of 90 maternal samples were used to evaluate the performance of a single separator model in enriching NRBC (data not published). The 90 samples were processed in six smaller groups, ranging from 6 - 34. Table 2, below, summarizes these six studies. The range of NRBC recovery is 113 - 23,250 per 15 ml of maternal blood. Assuming 35% of the NRBC were of fetal origin, the theoretical number of fetal cells per 15 ml of maternal blood ranges from 40 - 8,138. In this study, NRBC were detected in maternal circulation in 82% of the samples. The improved detection rate is due to selection of a single CFS-100 separator design.

Table 2. Detection of NRBC in samples separated with the CFS-100.

Study	Sample (n)	Samples w/NRBC	NRBC Range	Mean NRBC	Potential Fetal Cells
Study 1	13	13 (100%)	1,125 – 23,250	7,499	2625
Study 2	19	16 (84%)	113 – 5,650	1,487	520
Study 3	34	22 (65%)	156 – 5,850	1,021	357
Study 4	10	10(100%)	565 – 18,750	2,183	764
Study 5	8	8 (100%)	289 – 2,543	849	297
Study 6	6	5 (83%)	207 – 1,661	659	231
Average		**74 (82%)**	**113 – 23,250**	**2,231**	**799**

In the study described above, although the average number of fetal cells after the CFS is 799, the number of WBC at this step is unacceptably high to allow deposition of all the cells on 1-2 slides. This necessitates increasing both the recovery and purity of the fetal cells in order to approach a level of detection of 100%. At BioSeparations, Inc. the focus has been to increase the recovery of the NRBC. To that end, improvements in the CFS and IMAGESCAN, the cornerstones of the PDS system, have been made.

B. Results with the CFS-200

The PDS method steps were characterized and optimized for recovery of NRBC, for final purity of NRBC in relation to the contaminating maternal leukocyte and mature RBC, and for the identification of fetal cells. In order to monitor improvements in recovery at each step, a model system to mimic maternal blood was utilized. Samples were prepared by mixing a small amount of neonatal cord blood with peripheral blood from an adult of the opposite sex. Typically, 50,000 to 300,000 NRBC were spiked into 15 ml of the adult blood. The spiked sample with the fetal NRBC at a known concentration was processed with each PDS step being analyzed for recovery and purity. NRBC number was evaluated by counting benzidine-stained NRBC under brightfield illumination. These counts were then compared to the starting number of NRBC and to the number of white blood cells (WBC) and red blood cells (RBC) counted using a Coulter Z2 Particle Counter. Results were used to calculate the recovery and increase in purity of NRBC. Using the model system, improvements have been made and verified in each step of the PDS process. FISH with DNA probes for the X-and Y-chromosomes is used to monitor the labeling steps (fixation, slide preparation and FISH).

In the original design of the CFS-100, a time of 16 minutes was required for the cells to pass through the separator. Total cell recovery averaged between 30 and 50%, and NRBC recovery in all fractions was 21 ± 4%.

Significant design changes have been made, including alterations in the physical geometry of the separator, changes in the stabilization media, and changes in the electrophoresis buffer used. The physical changes have decreased the time needed for cells to pass through the separator to 1.5 minutes. The new CFS-200 also has benefits in terms of ease of manufacture, unit-to-unit reproducibility, and easier access for service and routine maintenance.

Using the model system, the optimized CFS-200 separator recovers 90 ± 13% (n = 4) of the fetal NRBC. This overall high recovery allows harvesting of 66% ± 16 (n = 4) of the NRBC in fraction 6 and 7 (refer to Figure 12). While this approach leaves approximately 33% of the NRBC behind (in fraction 8), it is justified by an additional 2-fold increase in purity due to the elimination of excess WBC (data not shown). In our model system, the CFS-200 provides a 3-fold increase in recovery of NRBC as compared

with the CFS-100, as shown in Figure 13. This should translate into a similar increase in recovery of fetal NRBC from maternal blood to be performed in this study.

The third step in the enrichment process is magnetic positive selection and lysis of contaminating mature RBC and WBC. For optimum detection when few fetal cells are present, all cells need to be contained on a single slide. A single slide requires four hours to scan and detect fetal cells. In spite of significant increases in NRBC recovery in previous enrichment steps, the number of nucleated maternal cells is still an issue for timely analysis of samples with very low numbers of fetal cells. Preliminary data indicate the desired purity can be obtained after the CFS with the use of monoclonal antibodies to NRBC. Treatment with a monoclonal antibody cocktail conjugated to paramagnetic fluid would result in minimal loss of fetal cells (10%), but a significant increase in purity, the maternal cell number being reduced one log (data not shown). The characteristics of these antibodies are described in Alvarez et al. "Development, characterization, and use of monoclonal antibodies made to antigens expressed on the surface of nucleated red blood cells." *Clin. Chem. 45, 1614-1620* (1999).

The use of monoclonal antibody positive selection on the CFS fractions containing over 70% of the fetal cells, has significant advantages over the same technique applied to density gradient processed blood. The concentration of cells, thus the amount of antibody required, is less in the CFS fractions. Selection is done on 2-3 ml of sample with total cell number of approximately 100 million and white cell number of 3-4 million. The CFS combined with density gradient centrifugation has increased the purity of the fetal cells by 300-fold or more. Since none of the antibodies identified to date are completely specific for the fetal NRBC, there is less probability of cross-reactive sites interfering with the blocking the antibody. The combination of CFS and magnetic positive selection will enable the sensitivity of detecting fetal cells in the circulation of pregnant women to approach 100%.

Carnoy's fixation and cell lysis removes the contaminating maternal RBC, allowing all cells to be deposited on a single slide. Preliminary studies indicate approximately 80% recovery of NRBC after fixation and slide preparation.

C. Labeling

Fixation and slide preparation improvements have contributed to the increased recovery of NRBC. Modifications in protocols, decreasing centrifugation times and

reduction in the number of steps have been made. Slide coatings have been compared and a standard procedure for cleaning and coating slides developed. These adjustments have decreased losses during fixation and slide preparation.

Modification of the ChromaHyb™ hybridization buffer has improved both hybridization efficiency and signal intensity following FISH. To provide evidence that NRBC nuclei hybridize with the same efficiency as WBC, CFS fractions were depleted of WBC using CD45 monoclonal antibody to remove leukocytes. The hybridization efficiency with X-and Y-DNA probes of the NRBC enriched fraction was 96.6 ± 4%. The mean hybridization efficiency of four samples was 96 ± 2%. FISH optimization also resulted in increased signal intensity. The increased intensity allows a more accurate determination of hybridization efficiency and use of a lower magnification, which decreases analysis time.

D. Detection

IMAGESCAN™ is a software package developed by BioSeparations, Inc. that aids in the detection of cytologic features during microscopic investigations. The software interacts with hardware, such as the stage, camera, filters, and nosepiece, during successive scans of a sample slide. During a scan, the software will adjust, both automatically and interactively with the user, hardware settings such as stage location and camera exposure time to facilitate the acquisition of cellular images of the highest possible quality. Once an image has been obtained, IMAGESCAN analyzes that image to locate cytologic features, such as cellular boundaries and chromosomes. The results of these analyses are stored in tabular form and are available to the user. In addition, smaller images of areas of potential importance, also known as "gallery images" are automatically stored to disk for subsequent evaluation by the user. IMAGESCAN also creates files that are placed on a web server so that studies can be monitored remotely via any web browser. When a study is completed, all data, images, and results associated with a study are automatically archived to disk. The user can review all analyses performed automatically by IMAGESCAN as well as further analyze the original images and related data, as desired.

RESULTS

The centrifugation and CFS steps yield an estimated recovery of 55 - 70% and 66%, respectively, of the starting number of NRBC. The use of monoclonal antibody positive selection allows for the further purification of the sample and achieves fetal cell recoveries of at least 90% of the NRBC in the CFS fractions. Recovery of approximately 80% of the NRBC is observed with the fixation step. FISH and IMAGESCAN each have a sensitivity of 95.5%.

Table 3 presents data demonstrating the depletion of contaminating maternal RBC and WBC and enrichment for NRBC during the progressive steps of the PDS. The post-lysis step results in a significant increase in purity with the removal of the mature WBC. As a result, 80% of the NRBC remain and can be centrifuged onto a single slide. Minimal losses of less than 10% are experienced during the FISH and IMAGESCAN steps. Initial cell numbers are based on Documeta Geigy (Diem and Lentner, 1970).

Table 3.

Progressive depletion of RBC and WBC during PDS processing of a maternal blood.

	Maternal Cells	
Cell Type	**WBC**	**RBC**
Initial Cell Number	1.0×10^8	6.2×10^{10}
Post Centrifugation	6.4×10^7	2.3×10^8
Post CFS	2.3×10^6	1.8×10^7
Post Antibody Selection	3.9×10^5	1.2×10^6
Post Lysis, FISH IMAGESCAN	3.5×10^5	0

Table 4, below, shows an estimated recovery of fetal and maternal cells by PDS beginning with 15 milliliters of maternal blood and different ratios of fetal to maternal cells. These data are based on the estimates of the number of fetal cells which cross the placenta into maternal circulation (Simpson and Elias, 1993; Bianchi et al., 1990).

Table 4.

Calculated recovery of fetal cells after each step of the PDS process.

	Fetal Cells		
Fetal/Maternal Cell Ratio	**$1/10^7$**	**$1/10^8$**	**$1/10^9$**
Initial Cell Number	7500	750	75
Post Centrifugation	4200	420	42
Post CFS	2772	277	28
Post Antibody Selection	2495	249	25
Post Lysis, FISH IMAGESCAN	1820	182	18

The frequency of $1/10^7$ and $1/10^8$ is commonly quoted as the expected range of fetal cells in maternal blood. Given that fetal cells have been detected in 50 - 80% of pregnant women (de la Cruz, 1998; Holzgreve et al., 1998b) one can predict that the fetal cells may be more rare than expected. Our data suggest that we can detect fetal cells in maternal blood at a frequency of 1 in 10^9 maternal cells, or as low as 18 fetal cells in the final step of processing. This sensitivity is consistent with detecting the range of fetal cells previously estimated from the study in Table 2. Using the data that 35% of NRBC in maternal blood are fetal in origin, it is estimated that as few as 40 fetal cells could be isolated with the PDS and detected on a single slide with IMAGESCAN.

The Prenatal Diagnostic System (PDS) of the present invention is carried out utilizing different fluorescent labels to cells for the detection of different potential targets. In general, probe or set of probes, such as the fluorescent γ-hemoglobin mRNA probe of Step 7, is applied to the samples. The cells are imaged, the fluorescent labels are removed, new labels are applied, and new cell images are obtained. Finally, the results are analyzed and reported during Step 13 of the Analysis/Reporting Task. The data are analyzed and reported in Step 20. The sequential steps employed in the PDS are the following:

1. Sample Collection

Approximately 20 ml of whole blood is collected from each subject in vacuum tubes containing ACD anti-coagulant.

2. Centrifugation

The blood is centrifuged on a Ficoll gradient for 40 minutes. The plasma and the erythrocyte portions are discarded and the remaining cells are washed twice. The cells are then resuspended in CFS running buffer containing 1% BSA to a final concentration of $3x10^7$cells/ml and a maximum of 10 ml is processed through the CFS instrument. Cell counts are performed using a Z2 Coulter Particle Count and Size Analyzer.

3. Charge Flow Separation

The CFS unit is operated under computer control with user-defined input at decision points. On startup, the unit is automatically flushed with water and electrolyte buffer. A solution of CFS running buffer with 0.5% BSA is added through the sample inlet port to condition the flowpath of the cells. The cells, which have been resuspended in CFS running buffer with 0.5% BSA, are loaded onto the instrument and the computer program is activated. The cells are pumped into the separation chamber, separated and collected into discrete fractions. When the run is complete, the CFS is prepared for the end of day shutdown. The cell fractions are centrifuged, and the cell pellets are then resuspended in the appropriate buffer prior to counting.

Monitoring of the separation can be performed in real time at BioSeparations, Inc. in Tucson. The CFS system has a network interface card (NIC) installed that enables the system to be connected to the Internet. While the CFS control program is running, the CFS current and voltage information will be sent as a Transmission Control Protocol/Internet Protocol (TCP/IP) packet to a web server, located at BioSeparations, Inc. in Tucson. The current and voltage information will be graphed onto an X-Y plot and displayed on a continuously updated web page. BioSeparations, Inc. personnel can then monitor the status of the CFS system via an Internet browser. This would allow the early detection of any separation problems indicated by abnormal current and voltage fluctuations.

4. Magnetic Selection

Monoclonal antibodies to early and late stage nucleated fetal red blood cells (Alvarez et al., 1999) have been directly conjugated to paramagnetic nanoparticles. These antibodies have been shown to have a low reactivity to white blood cells. Appropriate cell

fractions from the CFS are combined, incubated with the antibodies, and positively selected for NRBC.

5. Selective Lysis

Fetal NRBC are further enriched from contaminating maternal RBC by selective lysis of adult RBC using a modification of the method of Boyer et al. "Enrichment of erythrocytes of fetal origin from adult-fetal blood mixtures via selective hemolysis of adult blood cells: an aid to antenatal diagnosis of hemoglobinopathies," *Blood* 47, 886-897. (1976).

6. Slide Preparation and Fixation

The cells are applied to a slide using cytocentrifugation and are fixed in 3:1 ethanol/methanol.

7. FISH (γHb mRNA probes)

The use of γHb monoclonal antibodies covalently linked to fluorescent markers has in the past proven to be a valuable tool for the differentiation of female fetal cells from maternal cells (Martin et al., "Non-invasive fetal cell isolation from maternal blood," *Br J Obstet Gynaecol* 105, 576-583.1998, Oosterwijk et al., 1998), although the appearance of low concentrations (1-2%) of HbF in normal adult RBC represents a limitation of this method (Bajaj et al., "Ultra-rare-event detection performance of a custom scanning cytometer on a model preparation of fetal NRBCs," *Cytometry* 39, 285-294 (2000). Our preliminary data suggests that the probe to detect mRNA for the γ F globin chain of the fetal hemoglobin (HbF) can be used as an additional marker for fetal NRBC during FISH, and in some cases can be used to identify NRBC from female fetuses.

8. Scan and Generate Gallery

In Step 8, ImageScan systematically scans the entire sample disk of fluorescently labeled cells, field-by-field, using a 20x objective and a 2.5x internal lens located in the camera mount of the digital camera. This gives an overall magnification of 50x for the digital field images. The resolution of a 50x digital image approximately corresponds to that of a 200x optical image as seen by a human user. At this magnification, approximately 1050 fields are required to cover a sample disk with a 12mm diameter. The

scan proceeds field-by-field, moving horizontally and vertically across the entire area of the sample disk. An image of each field, referred to as a "field image", is captured as the scan proceeds. Parameters used in the scanning process, such as exposure time, are set at the beginning of the scan, based upon the characteristics of the sample and the desired objectives of the scan.

While ImageScan scans the disk as part of Step 8, it automatically searches the sample for γHb mRNA-labeled NRBC, each of which exhibits fluorescence in the cytoplasm. As seen in Figure 14, NRBC exhibit a red fluorescence in their cytoplasm as seen through a tetramethyl-rhodamine-isothiocyanate (TRITC) filter. Nuclei are identified by a bluish cast when stained with DAPI (4', 6-diamidino-2-phenylindole-dihydrochloride), and viewed through a dual-pass DAPI/TRITC filter. NRBC are identified automatically by ImageScan through the combined presence in one cell of red fluorescence in the cytoplasm and blue fluorescence in the nucleus of a single cell. The majority of the fluorescing NRBC are expected to be of fetal origin. However, a small percentage of maternal NRBC may also be labeled in the samples.

An image of the potential NRBC, called a "cell image", is captured automatically and stored into an image gallery. The field-by-field progress of the scan is displayed on password-protected web pages on the Internet through a web server located at BioSeparations, Inc. in Tucson. Image and data files are transferred from remote imaging sites to the web server in Tucson as each scan proceeds. All information related to the ongoing scan, including field and cell images, is available to a remote user via the Internet. These web pages will be used by scientists at BioSeparations, Inc. to monitor the scanning process in Tucson and to provide feedback to the local users at remote sites.

For each field in the scan during Step 8, ImageScan performs the following steps:

i) Position the slide so that the field is under the microscope objective.

ii) Focus the image.

iii) Switch to the TRITC filter.

iv) Identify cells expressing the γ-Hb gene within their cytoplasm. This is done by searching the field image for circular reddish objects. Note that it is generally accepted that 1-4% of maternal RBC express the γ-Hb gene, so maternal as well as fetal RBC will be identified in this step. The pattern recognition algorithms used by ImageScan might also identify some undetermined contaminants (false positives) in this step.

v) Switch to the DAPI/TRITC filter.

vi) Identify the nuclei in the image by searching for the bluish DAPI-stained nuclei.

vii) Identify the NRBC expressing the γ-Hb gene by correlating the objects found in steps iv and vi. The NRBC will be the red objects (the fluorescent γ-Hb mRNA probe) that contain a blue nucleus (DAPI-stained).

viii) Place an image in the gallery of each cell identified in step vii.

ix) Save an image of the entire field onto disk.

The photo-micrographs in Figure 14 show fluorescently labeled fetal NRBC, fetal RBC, maternal RBC, and WBC. The figure illustrates the reddish color of the cytoplasm within the fetal cells as compared to maternal RBC, which lack fluorescently labeled γ-hemoglobin mRNA. The expanded image (*i.e.* cell image) to the right represents the tile image that ImageScan captures automatically during scanning of fields and subsequent gallery generation. The green dot within the blue DAPI-stained nucleus of the fetal NRBC is a Y-chromosome that has been labeled with FISH.

9 Review Gallery Images/Select Cells

The user reviews the gallery of cell images created in Step 8 and selects the cells that he/she wishes to image during subsequent steps. During this review process, the user has several options to help identify true NRBC. The user can instruct ImageScan to automatically relocate the cell on the slide, then examine the cell under higher magnification or through different filters. Images of cells that the user has determined not to be NRBC will be removed from the gallery of cell images; these cells will not be imaged during subsequent steps (i.e., Steps 11, 15, and 18). An intuitive graphic-user interface (GUI) has been developed to facilitate the review process. At the end of Step 9, a revised gallery of cell images, chosen from the images obtained in Step 8, is produced with each image containing one or more true NRBC. This revised gallery is available for remote monitoring via the Internet at BioSeparations, Inc. and elsewhere.

10. FISH (X-, Y-probes)

The previously used slide is subject to a "ReFISH" procedure, in which the hybridized sample is washed in a 1:9 dilution of ChromaHyb Washstock and dehydrated in a series of ethanol washes (Thompson et al., 1997). The slide is air-dried and submitted to the ChromaHyb Pretreatment procedure which includes a 70% acid incubation to lyse fetal red blood cells and remove interfering cytoplasm for the following hybridization with directly labeled DNA probes for the X- and Y-chromosomes. The procedure itself is carried out according to manufacturers' instructions, as well as posthybridization washes in 1:80, 1:200 and 1:400 dilutions of the ChromaHyb Washstock. Before visualization the slides are counterstained with DAPI.

11. Image Selected Cells

In Step 11, the slide containing the cells with hybridized fluorescent X- and Y-probes is repositioned on the stage. The X-chromosome probe is a strongly fluorescent red label while the Y-chromosome probe is a more weakly fluorescent green label. For each cell selected in Step 9, ImageScan automatically positions the slide so that the cell is centered under the objective. A cell image is then captured using a triple-pass FITC/TRITC/DAPI filter at 20x magnification. A new gallery of cell images is obtained as the cells are relocated and imaged with the X- and Y-chromosome probes.

12. Review Gallery Images

The user reviews the gallery of cell images obtained from Step 11. As with the review process in Step 9, for any image in the galley, the stage can be repositioned so that the cell in question may be examined directly on the slide. Higher magnification objectives and different filters can be selected, as appropriate, for review of the images. For example, the weaker, green Y-probe may be more clearly seen through the more selective FITC/DAPI dual-pass filter instead of the FITC/TRITC/DAPI filter. The results of this review and those of Step 9 will be analyzed and report as part of Step 13. Slides are relabeled with different fluorescent DNA probes in Step 14 in order to study the ploidy of autosomal chromosomes 13 and 21.

13. Statistical Analysis/Final Report

The primary endpoint is the determined gender of the fetus. The statistic used to assess the performance of the PDS system for predicting fetal sex is the overall agreement proportion of PDS answers which agree with the amniocentesis or CVS gender determination. This proportion is accompanied by the 95% confidence interval estimate for agreement proportion, computed with exact binomial (Clopper-Pearson) methods.

14. FISH (13-, 21-probes)

As previously described (Step 10) the slide is submitted to another ReFISH procedure by using the wash protocol developed by Thompson et al. "New rapid FISH techniques using X and Y 13/21, 18 chromosome synthetic DNA probes," *In Vitro Cell Devel Biol-Animal* 33, 31A. (1997). Several authors were able to show that sequential FISH analysis can be performed between 3 to 9 times on the same slide without loss of specificity and intensity of the hybridization signals. (Wang et al., 1995; Dierlamm et al., "Successful use of the same slide for consecutive fluorescence in situ hybridization experiments,". *Genes Chrom Cancer* 16, 261-264 (1996); Thompson et al., 1997; Zhen et al., "Poly-fish: a technique of repeated hybridizations that improves cytogenetic analysis of fetal cells in maternal blood," *Prenat Diagn* 18, 1181-1185 (1998). The second hybridization is performed after a brief pretreatment procedure as outlined in step 8, with locus specific DNA probes for chromosomes 13 and 21, according to manufacturers' instructions after removing of prior hybridized probes using the same protocol as described in step 10. Following posthybridization washes the slide is counterstained with DAPI and the probes are visualized as described in Step 15.

15. Image Selected Cells

In Step 15, the slides containing the cells with hybridized fluorescent 13- and 21-probes are repositioned on the stage. Both of these probes are red and will be located on separate slides. As in Step 11, each cell in the revised gallery from step 9 is automatically centered under the objective and imaged at 20x magnification with the TRITC/DAPI, dual-pass filter. Only the red channel will be stored for each image. A new gallery of cell images is obtained from each slide as the cells labeled with either the chromosome-13 or the chromosome-21 fluorescent probe are relocated and imaged.

16. Review Gallery Images

The user reviews the gallery of cell images obtained from Step 15. This review process is similar to that of Step 12. However, in this case, the 13-chromosome and 21-chromosome probes are located on different slides since both probes are red. As with the imaging of selected cells in Step 15, TRITC/DAPI, dual-pass filter will be used for review. If necessary, higher magnification or use of a single-pass TRITC filter can be used to aid in recognizing the probes.

17. FISH (18- probes)

Once again the slide is submitted to the ReFISH procedure, by removing formerly bound DNA probe in a 1:9 wash dilution of ChromaHyb Washstock (Thompson et al., 1997). After the pretreatment procedure, the slide is hybridized with a directly labeled DNA probe for chromosome 18 according to manufacturers' conditions and visualized after the posthybridization washes by DAPI counterstain. The evaluation of the slide progresses as outlined in step 18.

18. Image Selected Cells

As in Steps 11 and 15, a new gallery of cell images is obtained from each slide, as the cells labeled with the chromosome-18 fluorescent probe are relocated and imaged.

19. Review Gallery Images

The user reviews the gallery of cell images obtained from Step 18. The review process in Step 19 is similar to that of Steps 12 and 16. However, in this case, cells are labeled with a single red probe for chromosome 18. As with the imaging of selected cells in Step 15, a TRITC/DAPI dual-pass filter will be used for review. If necessary, higher magnification or use of a single-pass TRITC filter can be used to aid in recognizing the probes. Results of this review will be analyzed and presented in a final report during Step 20.

20. Statistical Analysis

The prevalence occurrence rates of monosomy, disomy (expected result except for X and Y) and trisomy determinations for each of the chromosomes (X, Y, 13, 18 and 21) will be computed among the 180 subjects in this part of the study. Any noted occurrences

of additional polysomies beyond 3 copies will be tabulated. Mosaicism rates will be computed, and Clopper-Pearson confidence intervals for the occurrence rates will be established. The results will be compared to those obtained from the corresponding amniocentesis or CVS results, and an overall agreement rate computed. The occurrence rate of false positives and negatives will also be determined based on this comparison, with the same corresponding 95% confidence intervals determined.

By employing the foregoing PDS method steps, an integrated system of cell enrichment, labeling and detection is provided which permits users to image labeled cells using computer-controlled automated imaging, image analysis to detect and store labeled cells coordinates on each slide, and to permit internet-based access and remote control.

What is claimed is:

1. A prenatal diagnostic method, comprising the steps of: obtaining a maternal whole blood sample containing a population of nucleated fetal erythrocytes;
   a) enriching the population of nucleated fetal erythrocytes;
   b) labeling at least a portion of the nucleated fetal erythrocytes in the enriched sample obtained from step (b), with a detectable label;
   c) detecting nucleated fetal erythrocytes labeled with detectable label by creating digitized images of fields containing labeled nucleated fetal erythrocytes;
   d) processing the digitized images to create a plurality of coordinates positionally identifying labeled nucleated fetal erythrocytes in the digitized images of fields; and
   e) storing the digitized images and the plurality of coordinates onto a web-based internet server for remote access and manipulation of the stored digitized images.
2. The prenatal diagnostic method according to Claim 1, wherein the step of enriching further comprises the step of centrifugation to remove a major fraction of platelets and mature erythrocytes without substantial loss of nucleated erythrocytes.
3. The prenatal diagnostic method according to Claim 2, wherein the step of enriching further comprises the step of charge flow separation.
4. The prenatal diagnostic method according to Claim 3, wherein the step of charge flow separation further comprises the step of imparting a buffer flow through a charge flow separator having an flow orientation vector which is opposite to an applied electrical charge vector within the charge flow separator.
5. The prenatal diagnostic method according to Claim 1, wherein the labeling step further comprises the step of binding a fetal cell-specific antibody to nucleated erythrocytes in the enriched sample of nucleated red blood cells.
6. The prenatal diagnostic method according to Claim 5, wherein the fetal cell-specific antibody is conjugated to a magnetic bead.
7. The prenatal diagnostic method according to Claim 5, where the binding step is followed by binding a fluorescent label to nucleated erythrocytes in the enriched sample of nucleated red blood cells.

8. The prenatal diagnostic method according to Claim 1, wherein step d) further comprises the step of computer controlling a microscope to positionally control a microscope stage and microscope slide containing the sample of nucleated erythrocytes and computer controlling camera exposures to acquire digitizable images.

9. The prenatal diagnostic method according to Claim 1, wherein step d) further comprises the step of locating cytological features in detected cells and assigning and storing positional coordinates of the labeled features relative to the field.

10. The prenatal diagnostic method according to Claim 9, further comprising the step of defining field regions of interest to a user, digitizing images of the defined field regions and storing the defined field region digitized images onto a machine readable medium.

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(81) Designated States *(national)*: JP, US.

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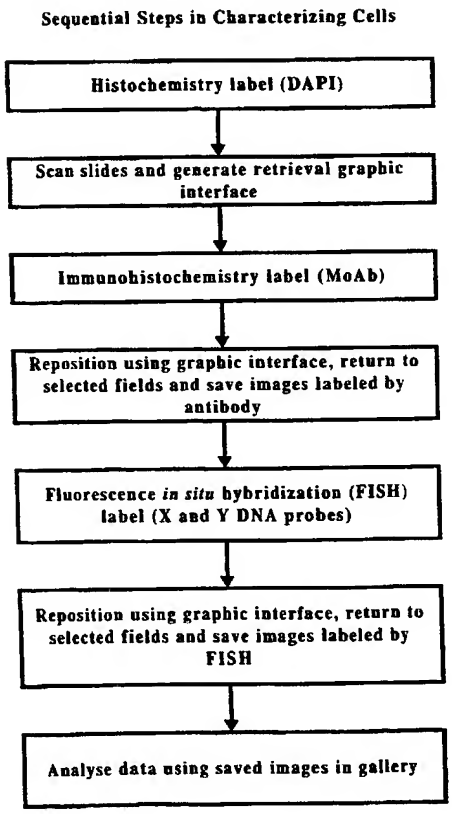
(54) Title: SYSTEM AND METHOD FOR PRENATAL DIAGNOSTIC SCREENING



(57) Abstract: A method prenatal diagnostic screening employing a system which integrates cell enrichment, labeling and detection steps. The labeling step characterizes cells using a combination of histochemical staining, antibody labeling and fluorescence *in situ* hybridization (FISH), and a method of detecting labeled cells using a computer imaging scanning system which detects labeled cells from immunohistochemical labeling and positionally compares the immunohistochemical labeled coordinates with a subsequently detected FISH label.

WO 00/75647 A1

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Sequential Steps in Characterizing Cells

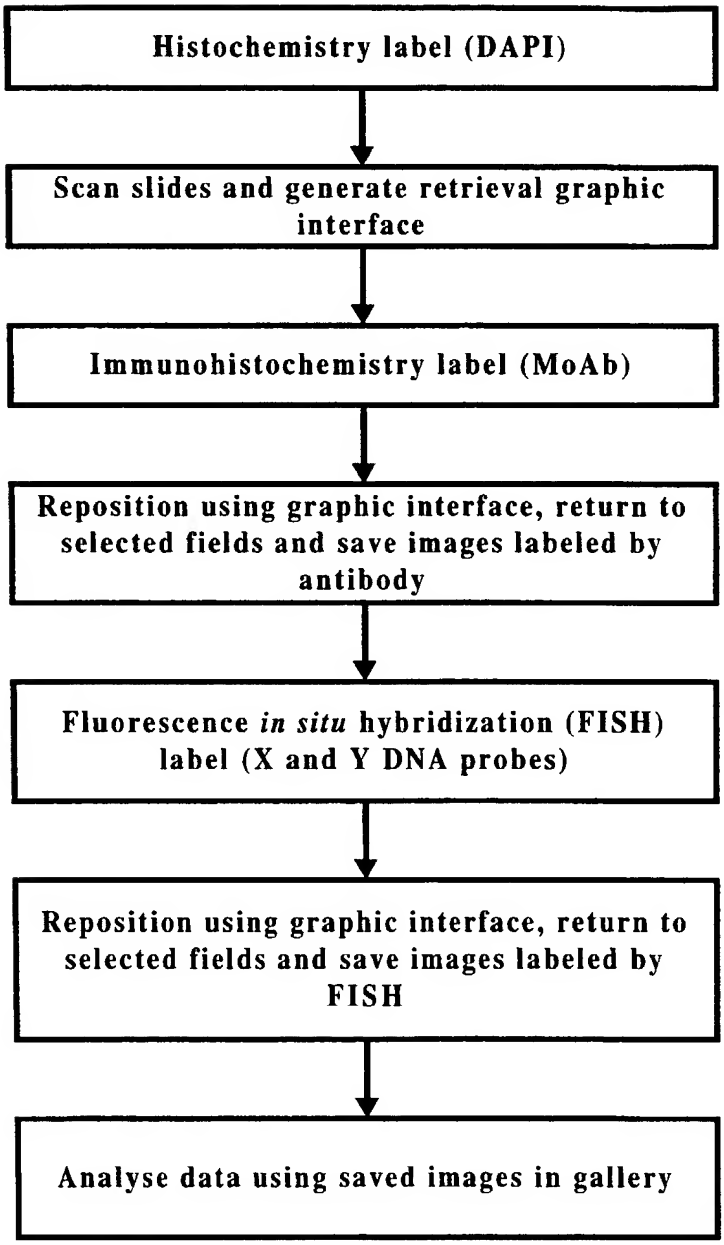


FIG. 1

FIG. 2

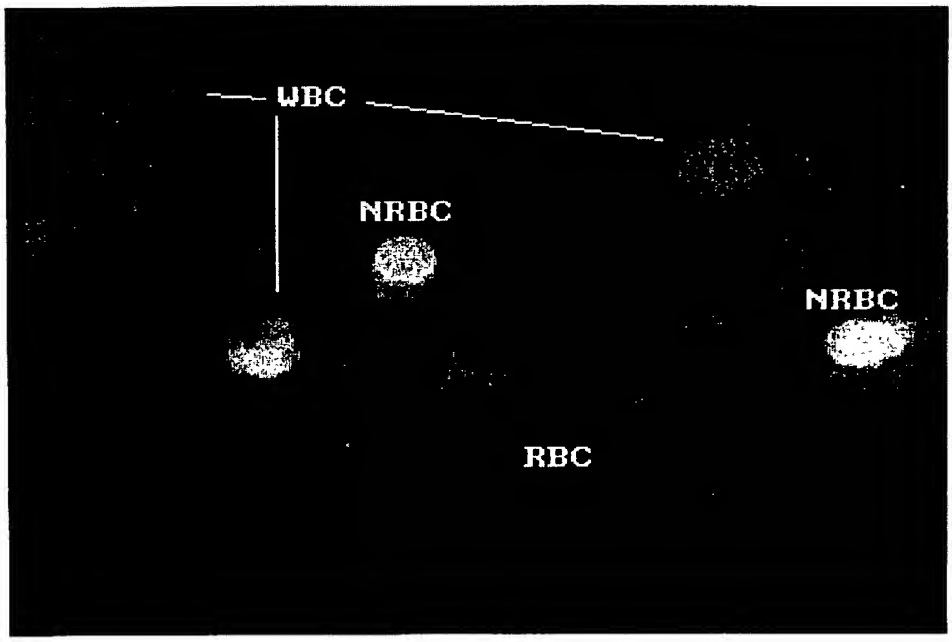
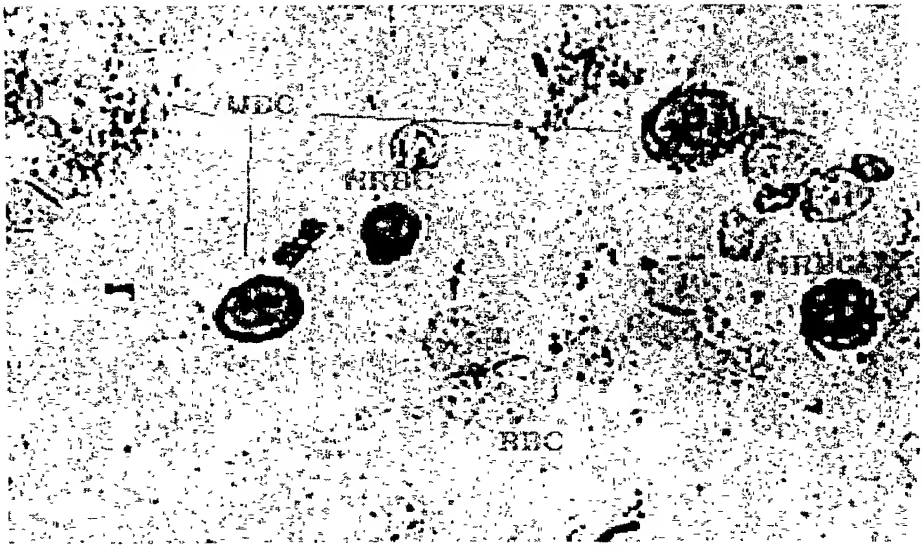


FIG. 3



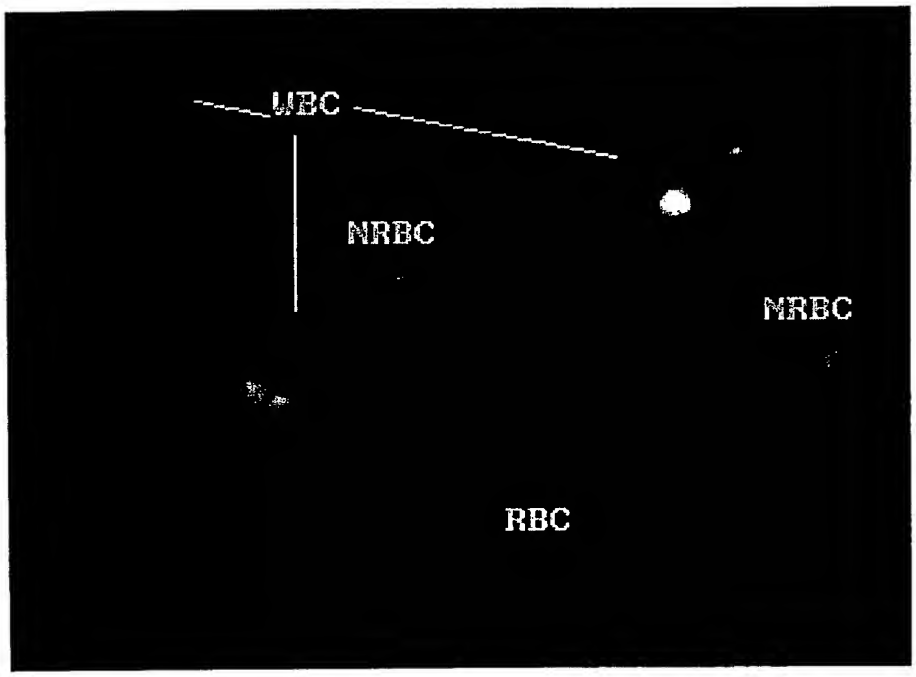


FIG. 4

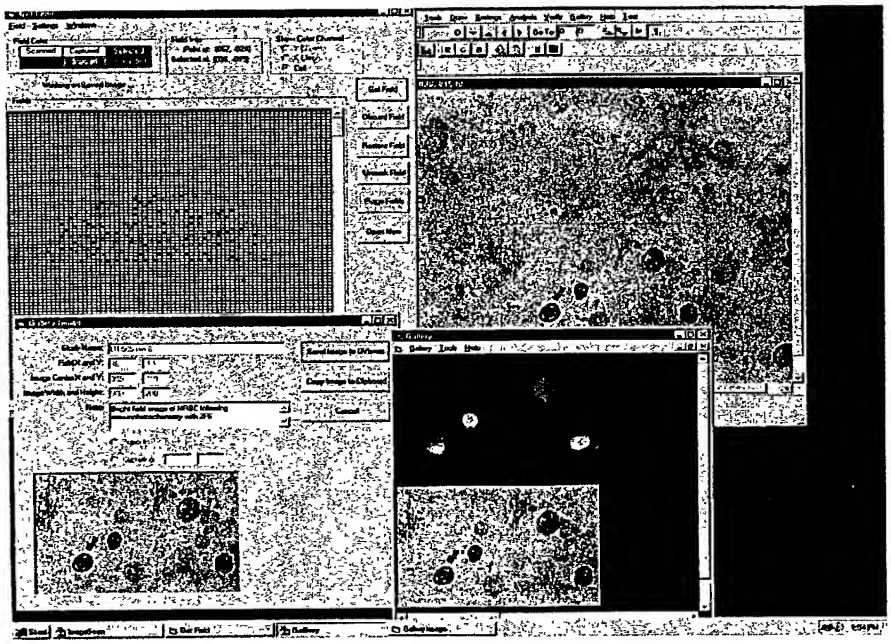


FIG. 5

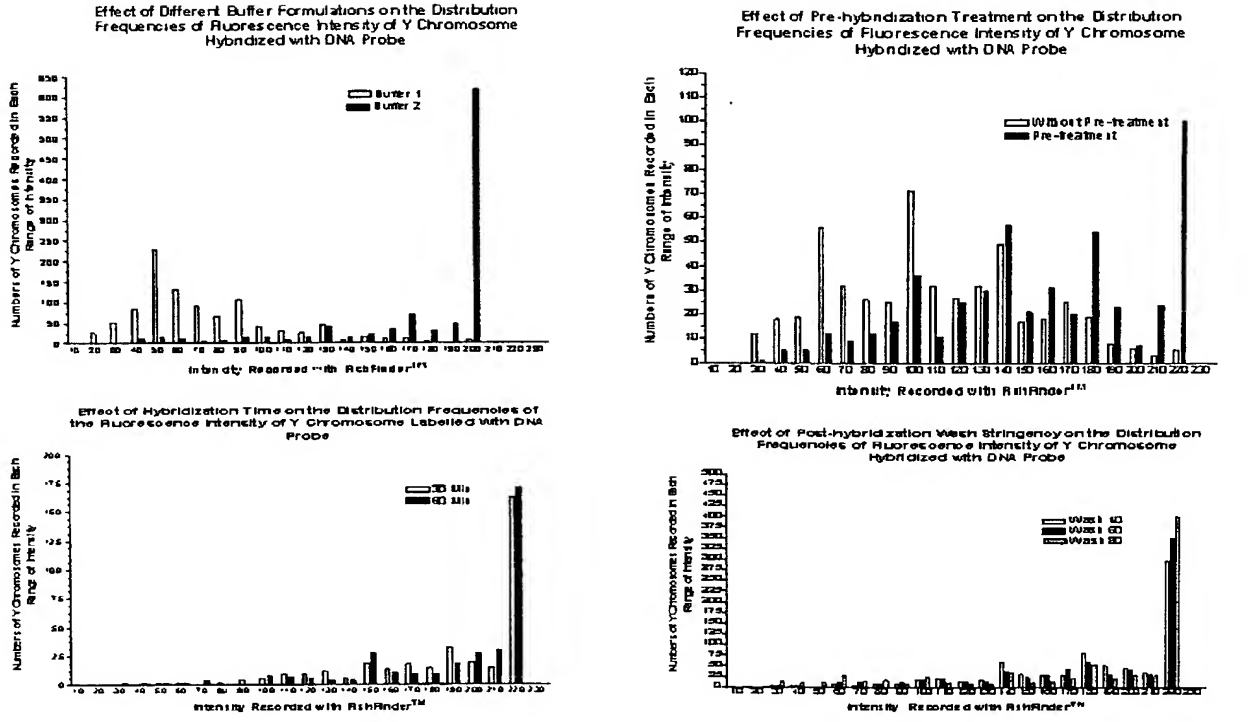

Effect of Different Buffer Formulations on the Distribution Frequencies of Fluorescence Intensity of Y Chromosome Hybridized with DNA Probe
Buffer 1
Buffer 2
Effect of Pre-hybridization Treatment on the Distribution Frequencies of Fluorescence Intensity of Y Chromosome Hybridized with DNA Probe
Without Pre-treatment
Pre-treatment
Effect of Hybridization Time on the Distribution Frequencies of the Fluorescence Intensity of Y Chromosome Labelled with DNA Probe
Effect of Post-hybridization Wash Stringency on the Distribution Frequencies of Fluorescence Intensity of Y Chromosome Hybridized with DNA Probe

FIG. 6

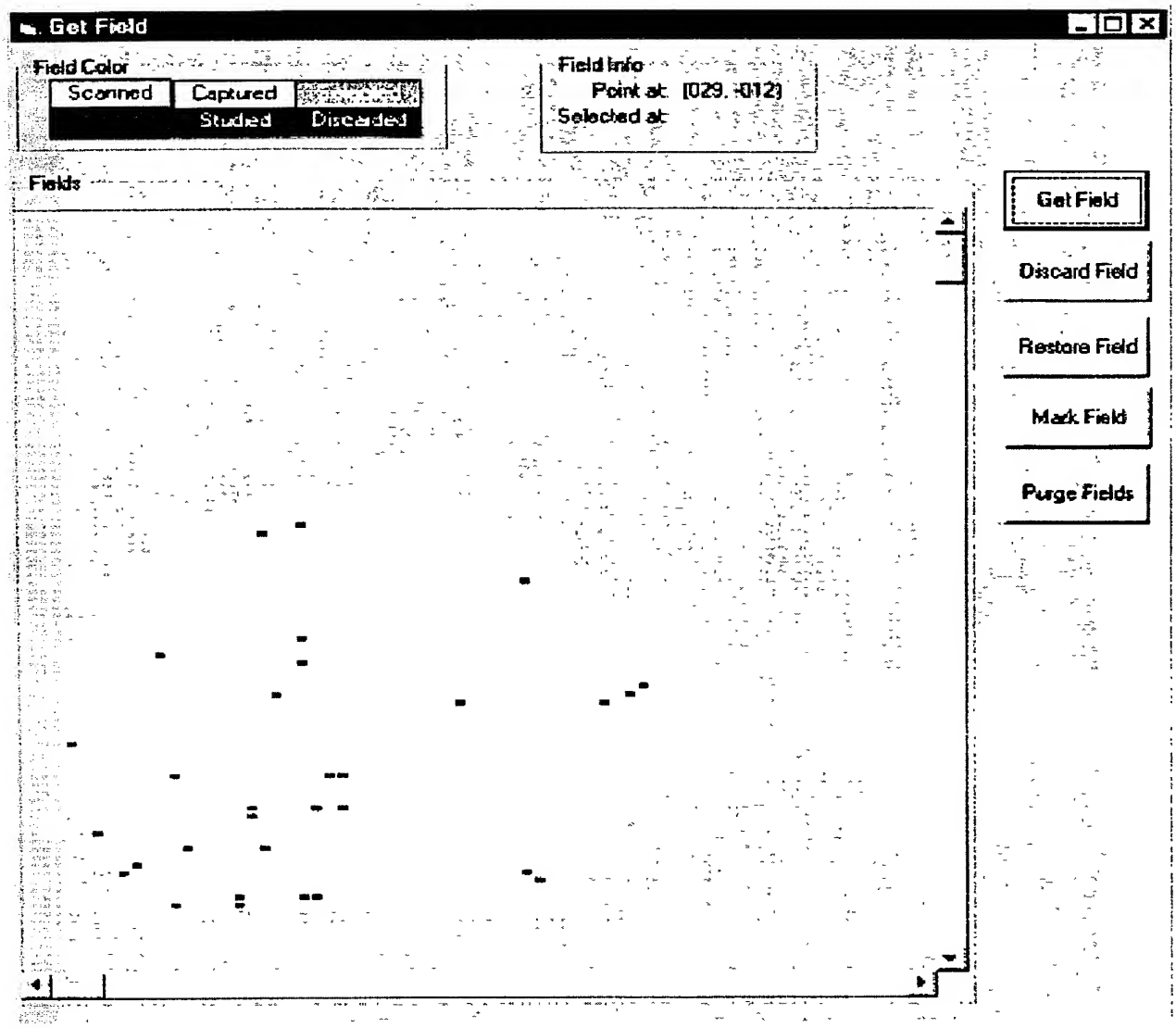

Get Field
Field Color
Scanned
Captured
Studied
Discarded
Field Info
Point at: [029, -012]
Selected at:
Fields
Get Field
Discard Field
Restore Field
Mark Field
Purge Fields

FIG. 7

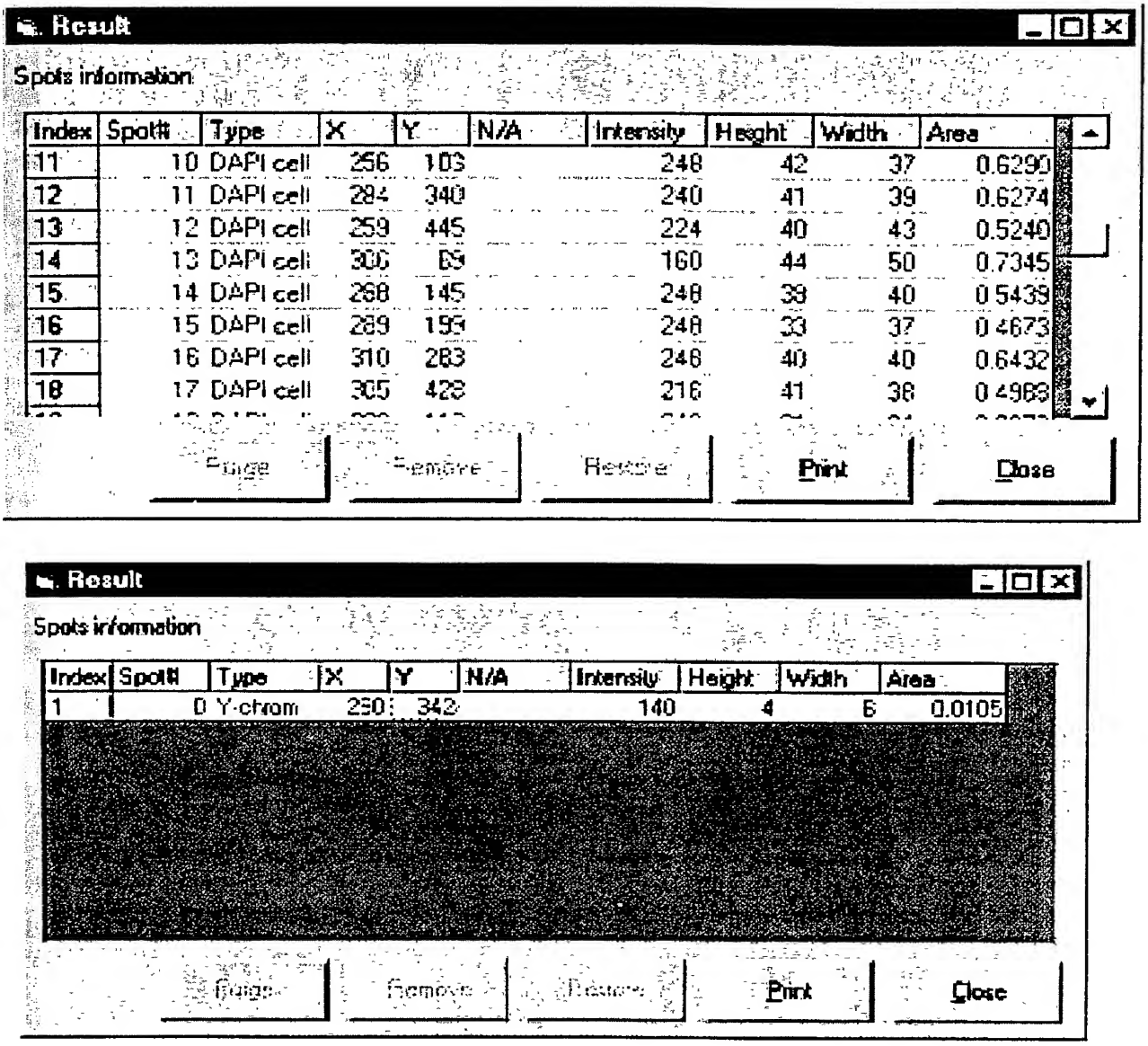

Result
Spots information
Index Spot# Type X Y N/A Intensity Height Width Area
11 10 DAPI cell 256 103 248 42 37 0.6290
12 11 DAPI cell 284 340 240 41 39 0.6274
13 12 DAPI cell 259 445 224 40 43 0.5240
14 13 DAPI cell 300 89 160 44 50 0.7345
15 14 DAPI cell 268 145 248 38 40 0.5438
16 15 DAPI cell 289 199 248 33 37 0.4673
17 16 DAPI cell 310 283 248 40 40 0.6432
18 17 DAPI cell 305 428 216 41 38 0.4988
Purge
Remove
Restore
Print
Close
Result
Spots information
Index Spot# Type X Y N/A Intensity Height Width Area
1 0 Y-chrom 230 342 140 4 6 0.0105
Purge
Remove
Restore
Print
Close

FIG. 8

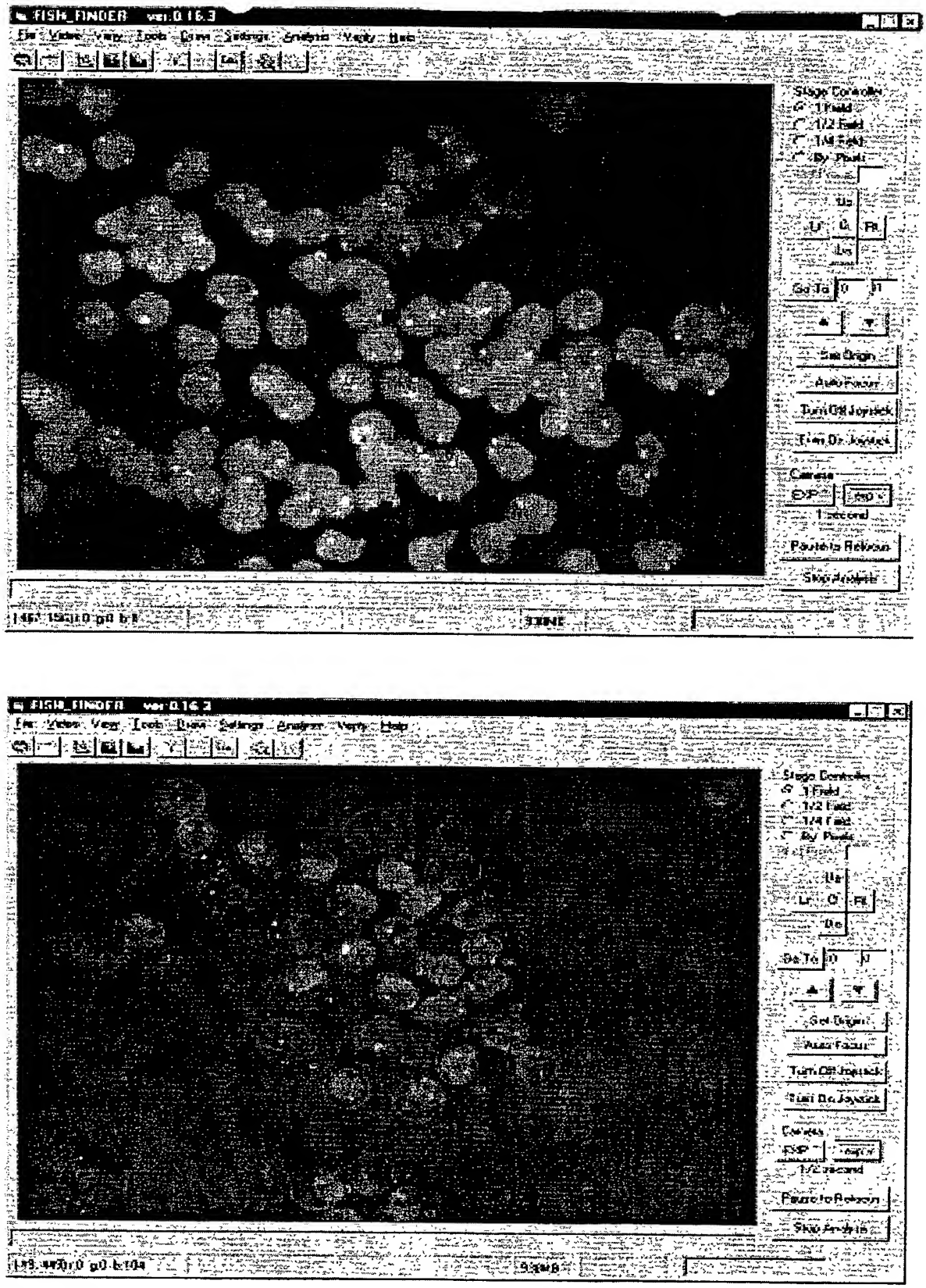


FIG. 9

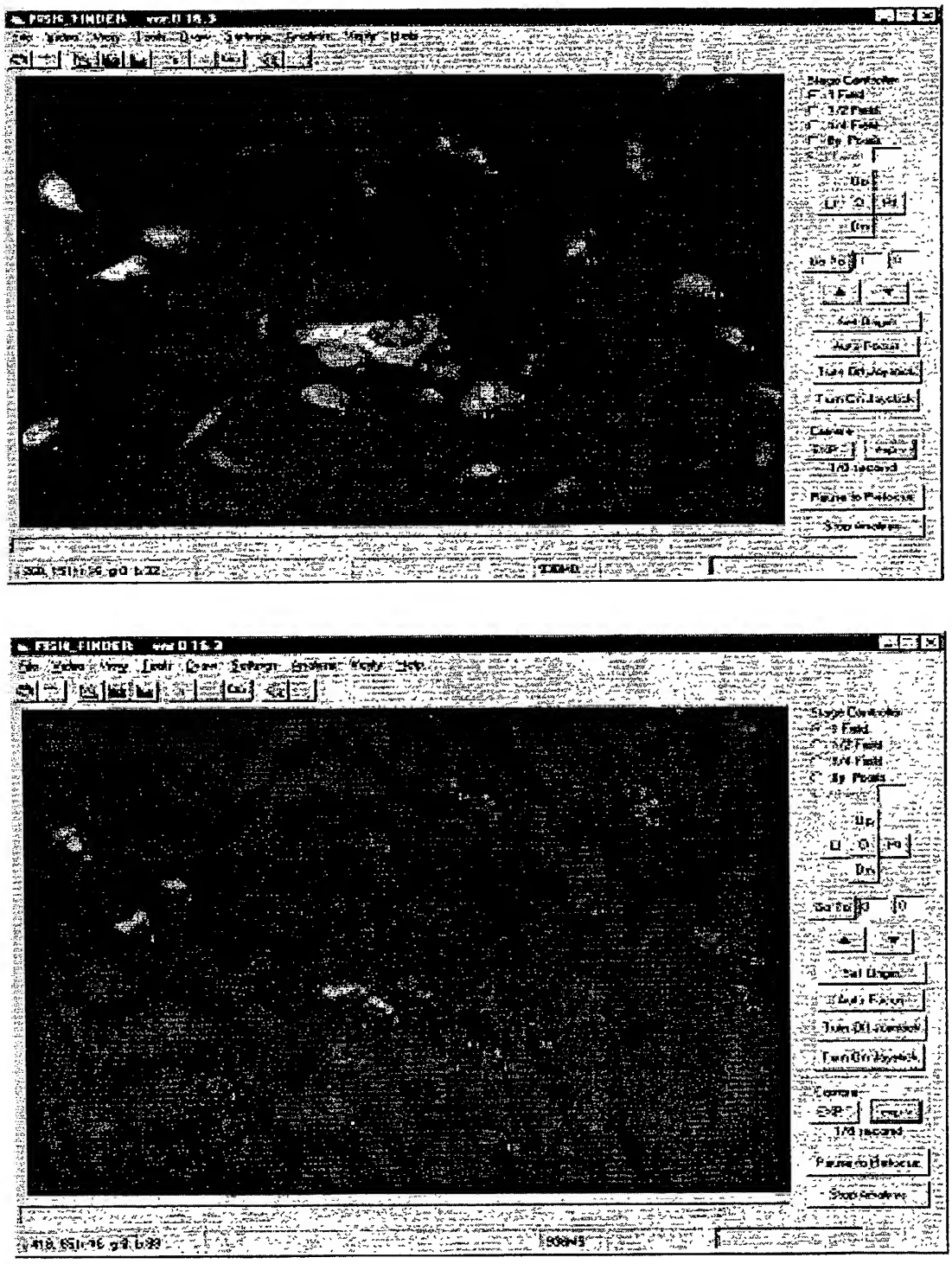


FIG. 10.

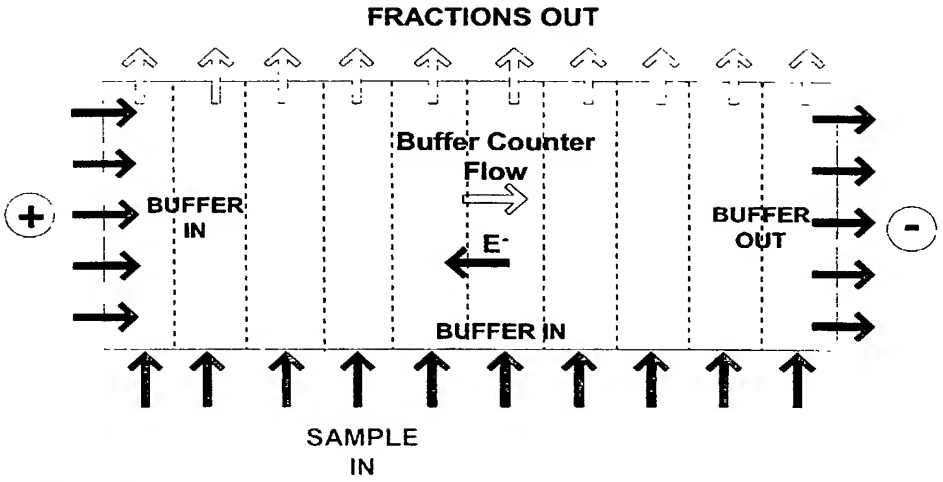

FRACTIONS OUT
Buffer Counter Flow
BUFFER IN
E-
BUFFER OUT
BUFFER IN
SAMPLE IN

Fig. 11

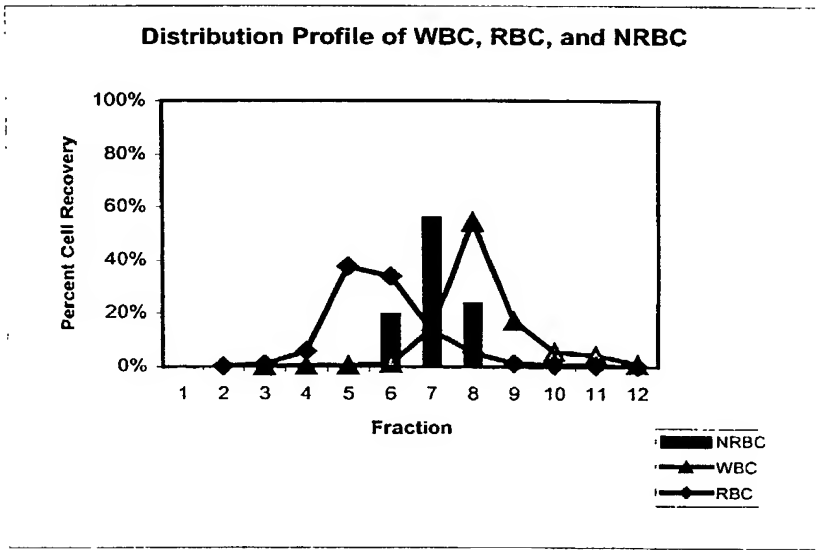

Distribution Profile of WBC, RBC, and NRBC
Percent Cell Recovery
100%
80%
60%
40%
20%
0%
1 2 3 4 5 6 7 8 9 10 11 12
Fraction
NRBC
WBC
RBC

Fig. 12

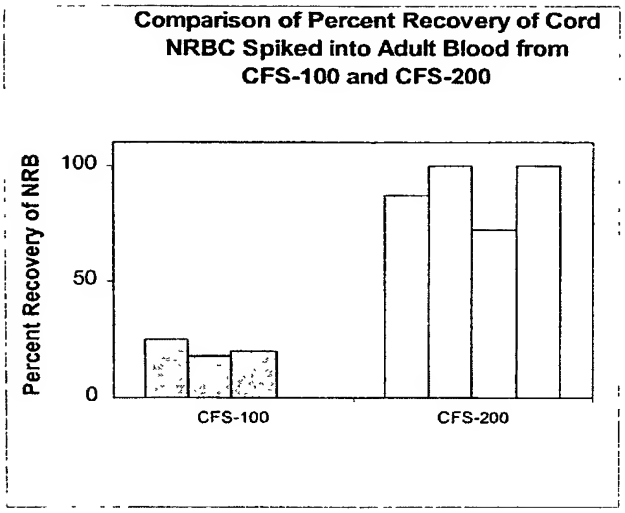
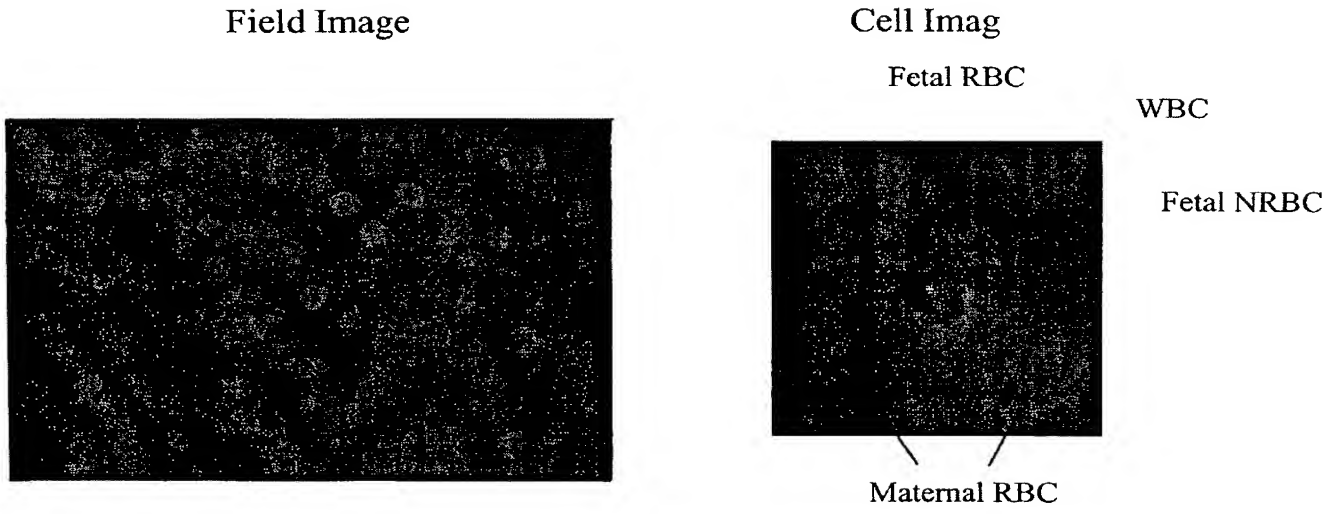

Comparison of Percent Recovery of Cord NRBC Spiked into Adult Blood from CFS-100 and CFS-200
Percent Recovery of NRB
100
50
0
CFS-100
CFS-200

Fig. 13

Fig. 14


Field Image
Cell Imag
Fetal RBC
WBC
Fetal NRBC
Maternal RBC

10009085 .071102

DECLARATION FOR PATENT APPLICATION

Docket No. 6033-001

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name, I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled **SYSTEM AND METHOD FOR PRENATAL DIAGNOSTIC SCREENING** the specification of which:

** check one ** [ ] is attached hereto.
[X] was filed on June 5, 2000
as Application Serial No. 10/009,085
and was amended on
(if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

60/137,692	June 4, 1999	PROVISIONAL
APPLICATION SERIAL NO.	FILING DATE	STATUS PATENTED, PENDING, ABANDONED
PCT/US00/15565	June 5, 2000	NATIONAL STAGE
APPLICATION SERIAL NO.	FILING DATE	STATUS PATENTED, PENDING, ABANDONED

POWER OF ATTORNEY

As a named inventor, I hereby appoint the following attorneys to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

David G. Rosenbaum, Reg. No. 31,872 Thomas S. Kim, Reg. No. 51,009.

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875 North Michigan Avenue
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Direct Telephone Calls to:
David G. Rosenbaum (312) 397-0303

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Inventor's signature _____ Date 6/15/02

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0696

DECLARATION FOR PATENT APPLICATION
Continued

Docket No. 6033-001

9-00

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Inventor's signature Delia R. Bethell Date 6-26-02

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3-00

Full name of joint inventor: Wen-Jen Ko

Inventor's signature _____ Date _____

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DECLARATION FOR PATENT APPLICATION
Continued

Docket No 6033-001

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Full name of joint inventor ~~Won Jon Ko~~ Wen-Jeng Ko

Inventor's signature Wen-Jeng Ko Date 7/9/2002

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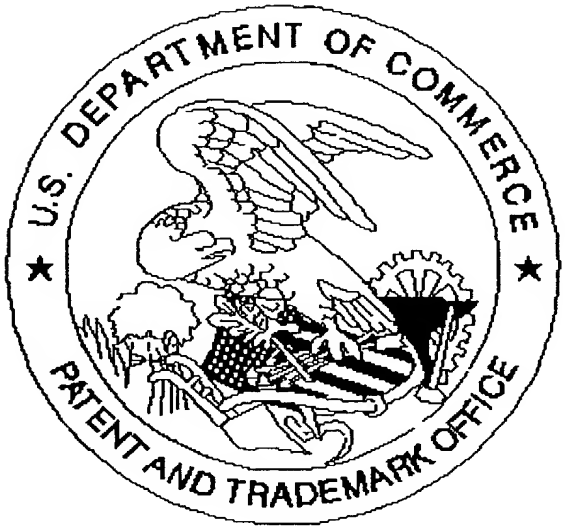
Citizenship: U S.A.

Post Office Address Same as above.

United States Patent & Trademark Office

Office of Initial Patent Examination -- Scanning Division



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